POLYSACCHARIDE HAPTENS FROM *TORULA HISTOLYTICA*

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Torula histolytica is a yeast-like organism which multiplies by budding, does not produce mycelia and rarely ferments carbohydrates. It may be pathogenic for man and is most frequently associated with infection of the central nervous system and lungs. The organism is characterized by a capsule, the thickness of which may be very much greater than the cell itself. The studies of Kligman (1947) indicated that the capsular material was mainly polysaccharide, but he failed to demonstrate any antigenicity for rabbits or mice with the strains he was using.

A locally isolated strain of *Torula histolytica* was found to be capable of stimulating antibody production in rabbits to a higher titre than those recorded in the literature. This strain was therefore selected as a suitable one to use in the attempt to isolate antigenically-reactive polysaccharide. The yeast fractionation was carried out in conjunction with investigations (not yet submitted for publication) on bacterial polysaccharides.

MATERIALS AND METHODS.

Isolation and Properties of Yeast.

The strain of *Torula histolytica* was isolated in pure culture from the cerebro-spinal fluid (on 5 occasions covering a period of 18 days) and at post-mortem examination from the meninges and lungs of a patient who died on 9/3/45. The yeast grew well on Sabouraud's medium and produced mucoid cream-coloured colonies after 3 days' incubation at 22° C. Further incubation resulted in a deepening in colour of the pigment to yellow or light brown and an increase in the size of the colonies, some of which were 15 mm. in diameter. Occasionally the growth was so prolific that it eventually dropped on to the lid of the petri dish. The organism fermented no carbohydrates. It was pathogenic for mice after intraperitoneal and intracerebral inoculation. Mice died from four to twenty-one days after receiving 0·1 ml. of a saline suspension of the viable cells. Films from mouse-brain of infected animals showed torula cells with a capsule the thickness of which was two to three times the diameter of the cell. In culture the capsule was smaller.

Preparation of Cultures for Fractionation.

The culture-medium was of the following composition: glucose, 4·0 gm.; neopeptone, 1·0 gm.; agar (Davis Gelatine Pty. Ltd.), 2·0 gm.; distilled water, 1,000 ml.

The medium was dispensed and autoclaved in large test tubes which were used as roll-tubes by the method described by Stanley (1949). The tubes were inoculated from a saline suspension containing approximately 10^7 organisms/ml. and incubated for five days at 22° C. The mucoid growth accumulated at the bottom of the test tubes after four days' incubation. On the fifth day the growth was washed off in distilled water and the organisms recovered by centrifugation.

Preparation of Antiserum.

Antiserum was prepared in the rabbit a few weeks after the isolation of the organism. The immunizing agent consisted of a washed suspension of torula cells in 0·85 p.c. saline. This was steamed for 20 minutes and standardized to tube No. 9 on Burroughs Wellcome opacity standards. The rabbit received (intravenously) 2·0 ml. of this suspension every 48 hours until 12·0 ml. had been administered and was bled 6 days after the last injection. The organisms were completely agglutinated at a serum dilution of 1/512.

EXPERIMENTAL.

Isolation of Polysaccharide.

Method (1). The centrifuged deposit containing the torula cells was taken up in normal saline to give a thick creamy suspension. The material was placed in a nickel crucible and the cells disrupted by freezing and thawing forty times, using dry ice in acetone as a freezing mixture. Microscopic and cultural examination showed that not all the cells were disrupted by this means. The mucoid material in the crucible was extracted three times with water and the deposit discarded. A trace of sodium acetate and two volumes of ethanol were added and the precipitate allowed to settle overnight. The precipitate was extracted twice with distilled water and the deposit discarded. The aqueous extract was precipitated twice more with two volumes of ethanol in the presence of a trace of sodium acetate. The resulting aqueous solution was extracted four times with chloroform-amyl alcohol mixture by the method of Sevag to remove traces of protein. The resulting solution was precipitated once more with two volumes of ethanol and allowed to stand overnight at room temperature. The precipitate was washed in ethanol and ether and dried (fraction P).

Method (2). In this method the yeast cells were disrupted by the process outlined by Kligman (1947). The deposited cells were taken up in water to form a thick suspension. An equal volume of normal hydrochloric acid was added and the solution heated to 75° C. for ten minutes, then cooled. The iodine test for starch was negative (see Mager, 1947). The solution was centrifuged and the deposit set aside for further extraction. The clear supernatant fluid was neutralized with caustic soda and a few crystals of sodium acetate and two volumes of ethanol were added. An immediate stringy precipitate (fraction S) was collected on a glass stirring rod. The remaining alcoholic solution was allowed to stand overnight at room temperature when it yielded a flocculent precipitate (fraction F). Precipitates S and F were recovered, washed in ethanol and redissolved in water. Both viscous aqueous solutions were repeatedly extracted with chloroform-amyl alcohol mixture. Each was then precipitated with two volumes of ethanol and the precipitates washed in ethanol and ether and dried.

The centrifuged deposit from the hydrochloric acid extraction of the organisms was taken up in 5 p.c. sodium hydroxide at 70° C. After further centrifugation the supernatant fluid was neutralized with hydrochloric acid and material precipitated by two volumes of ethanol was then obtained. The stringy precipitate that formed immediately upon the addition of

ethanol was dissolved in distilled water, treated with chloroform-amyl alcohol and then reprecipitated with ethanol and dried (fraction N).

Chemical tests on these fractions are recorded in Table 1.

TABLE 1.

Fraction	Biuret test	Molisch test	1. Bial's test	2. Dische test	3. Phosphorus
P	—	+	+	—	—
S	—	+	+	—	+
F	—	+	+	—	—
N	—	+	+	—	—

1. Modification of Mejbaum (1939).
2. Cysteine-sulphuric acid reaction for desoxyribonucleic acid (Dische, 1944).
3. Molybdivanadate method of Simonsen *et al.* (1946).

Serology.

Each fraction gave an immediate ring test with antiserum prepared (in 1945) against the whole cell. Serial double dilutions of the fractions were made in normal saline, and put up against the antiserum at a dilution of 1/80. The results shown in Table 2 were recorded after two hours at 55° C. Included in the Table is the result obtained with ribonucleic acid prepared from the same strain of *Torula histolytica* by the method of Johnson and Harkins (1929). The ribonucleic acid did not give a ring test with the antiserum.

TABLE 2.

Fraction	Dilutions of fraction × 10–3								
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
P	+	+	+	+	+	+	+	+	—
S	+	+	+	+	+	+	+	+	—
F	+	+	+	+	+	+	—	—	—
N	+	+	—	—	—	—	—	—	—
RNA	—	—	—	—	—	—	—	—	—

+ = precipitation. RNA = ribonucleic acid.

Attempts were made to immunize rabbits against the polysaccharides. For this purpose solutions containing 10 mg./ml. were made up in normal saline. Each rabbit received intravenously 10 mg. in 1 ml. saline every 48 hours for fourteen days—a total of 70 mg. With these doses no toxic effects were observed. After a further period of seven days the rabbits were bled and the serum tested by precipitation and complement fixation methods for the presence of antibodies to the polysaccharides. The results were uniformly negative.

Haemagglutination Tests.

Experiments were carried out to determine whether the fractions possessed haemagglutinating activity for the red cells of the fowl, guinea-pig, sheep and horse. Serial double dilutions of the test substance in saline were made, the initial concentration in the first tube

being 0·25 mg./ml. An equal volume of 0·3 p.c. red cells was added and the results (recorded in Table 3) read after one and two hours at room temperature.

TABLE 3.

Fraction	Dilution × 10^{-3}							
	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
P	—	—	—	—	—	—	—	—
S	+	+	+	+	—	—	—	—
F	—	—	—	—	—	—	—	—
N	—	—	—	—	—	—	—	—
RNA	+	+	+	+	+	+	±	—

+ = haemagglutination. RNA = ribonucleic acid.

The titres obtained with sheep, horse and guinea-pig red cells were almost identical and are omitted from the Table.

Mouse Protection Tests.

Forty-eight mice were given 1 mg. of polysaccharide by the intraperitoneal route every 48 hours until 4 mg. had been given. Twelve mice were used for each fraction and no toxic reactions were observed. After an interval of seven days each mouse was inoculated by the intraperitoneal route with 0·1 ml. of a saline suspension containing approximately 10^7 viable torula cells/ml. A group of twelve normal mice inoculated at the same time served as a control. The results shown in Table 4 indicate that under the conditions of the experiment no significant protection was achieved by prior administration of the polysaccharides.

TABLE 4.

Fraction	Number of mice dying during intervals indicated days											Survival	Total
	0–2	2–4	4–6	6–8	8–10	10–12	12–14	14–16	16–18	18–20	20–22		
P			4	3		1		1		1		2	12
S			1	2	3	2	1		2			1	12
F		2	4		1			2			1	2	12
N		2	2	2			3					3	12
—		1	5	1					1		1	3	12

Haemopoietic Response.

Fractions were injected intravenously into rabbits and total and differential leucocyte counts performed at 4, 24, and 48 hours after injection. The only significant change was an increase in the number of neutrophils between 4 and 24 hours after injection (see Table 5). No leucopenia was evident although some highly toxic bacterial polysaccharides are capable of causing an immediate leucopenia followed by a profound leucocytosis (unpublished observations).

TABLE 5.

Fraction (10 mg. in 1·0 ml. saline)	Time of count	Total leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*	
			p.c.	absolute	p.c.	absolute	p.c.	absolute
P	preinjection count	8,100	56	4,500	44	3,600	—	—
	after 3 hours	17,400	17	3,000	82	14,200	1	200
	after 24 hours	10,400	29	3,000	71	7,400	—	—
	after 48 hours	12,000	34	4,100	64	7,700	2	200
S	preinjection count	12,500	58	7,300	41	5,100	1	100
	after 3 hours	15,800	42	6,600	58	9,200	—	—
	after 24 hours	12,800	52	6,700	43	5,500	5	600
	after 48 hours	11,000	48	5,300	50	5,500	2	200
F	preinjection count	10,500	70	7,400	29	3,000	1	100
	after 3 hours	18,600	48	9,900	48	9,900	4	800
	after 24 hours	17,200	52	8,900	48	8,300	—	—
	after 48 hours	12,600	54	6,800	45	5,700	1	100
N	preinjection count	14,100	68	9,600	30	4,200	2	300
	after 3 hours	36,200	40	14,500	60	21,700	—	—
	after 24 hours	16,000	32	5,100	67	10,700	1	200
	after 48 hours	13,800	47	6,500	50	6,900	3	400

* per cubic millimeter.

At the same time that leucocyte counts were performed samples of blood were taken for blood sugar estimation by the Hagedorn-Jensen method and the temperature of the animals taken per rectum. There was no significant alteration in either of these features.

DISCUSSION.

A recent article on the capsular swelling (*Quellung* reaction) of *Torula histolytica* has appeared (Neill *et. al.*, 1949) in which it was shown that the capsular swelling of torula cells in the presence of antiserum did not occur when the antiserum was absorbed with the yeast polysaccharide. Neill and his colleagues also demonstrated that the torula polysaccharide (in high dilution) would precipitate with torula antiserum. No record was made of any attempt to determine if the polysaccharides were capable of stimulating the production of antibodies. It does not seem justified, therefore, to refer to such torula polysaccharides as antigens as done by Neill *et al.* Although the method of preparation of one of the two polysaccharides used was not given there is no evidence to suggest that the material with which they were working would not behave in a manner similar to the haptens described in this communication.

The work of Kligman (1947) has been confirmed in that it has been possible to isolate a non-antigenic polysaccharide from *Torula histolytica*. Kligman was unable to demonstrate any serological response in mice or rabbits with living cells, heat-killed cells, phenol-killed cells or decapsulated cells. He also obtained negative results in the attempt to produce antibodies using adjuvants

such as paraffin oil as outlined by Freund and Bonanto (1944). He was, therefore, unable to examine the serological activity of his polysaccharide with antiserum prepared against the cell.

It has been shown that the administration of polysaccharides of *S. typhimurium*, as well as stimulating antibody formation, caused toxic reactions, leucopenia followed by leucocytosis and changes in the carbohydrate metabolism of the experimental animal (Raistrick and Topley, 1934; Delafield, 1934; Martin, 1934; Cameron *et al.*, 1940). On the other hand, the evidence submitted in this paper shows that the administration of torula polysaccharides did not stimulate antibody formation, caused no demonstrable toxic reaction, did not produce leucopenia, and was unable to cause significant alteration of the blood sugar level in rabbits.

One of the methods (a modification of Kligman's) used in the extraction of the polysaccharide was exceedingly drastic and involved the use of acid and alkali. This was unfortunate but it was found to be the only method which completely disrupted the yeast cell. The difficulties associated with the disintegration of torula cells have not yet been overcome and other methods, both mechanical and chemical, have met with little success. This problem is an important one as it is evident from Table 2 that some serological activity is destroyed by alkali. Surprisingly enough the fractions isolated with the use of acid were no different serologically from that fraction obtained after disintegration of the organisms by freezing and thawing. It has not been determined whether the haptens isolated could be made antigenic by combination with protein, although Stoddard and Cutler (1916) presented evidence which led them to postulate that capsular material interfered with antibody production.

Mager (1947) claims to have isolated from a torula strain a capsular polysaccharide which stained blue with iodine and was apparently a mixture of starch and pentosan. This result was not confirmed with the strain of *Torula histolytica* used in the present investigations.

The titre of the rabbit antiserum prepared against the intact yeast cell was higher than those recorded in the literature. In rabbits, Sheppe (1929) obtained no significant titre, Rappaport and Kaplan (1926) a titre of 1/80, Benham (1935) a titre of 1/160, Hoff (1942) a titre of 1/280 and Cox and Tolhurst (1946) a titre of 1/128. As much the same methods were used in each case the differences recorded are probably due to strain variation.

A point of interest is the haemagglutinating activity of fraction S and the ribonucleic acid preparation. It is likely that this property of the polysaccharide is due to contamination with the nucleic acid—a fact which is not surprising when one considers the method of alcohol precipitation used and the finding of Alberty and Heidelberger (1948) that pneumococcal polysaccharide may contain nucleic acid.

SUMMARY.

The isolation and properties of some polysaccharide fractions of *Torula histolytica* have been described. These fractions behave as haptens and possess low toxicity for rabbits.

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EASE OF RELEASE OF HISTAMINE FROM THE LUNGS OF GUINEA-PIGS

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It has already been shown that previous experience influences the ease of release of histamine from the isolated perfused lung of the guinea-pig following the injection of trypsin (Trethewie and Day, 1948). In this paper an endeavour has been made to determine if there is any inherent difference in reactiveness as assessed by the release of histamine following such trypsin injection in guinea-pigs whose lungs are perfused at two different age groups, namely a few days after birth and during the third month of life. A considerable difference was obtained in the amount of histamine released (calculated on a weight basis for the animal) when trypsin was injected in proportion to the weight of the animal.

METHOD.

Selection of Animals.

From a constant breeding source pregnant guinea-pigs were selected that were about to drop their young. As the animals were born they were numbered consecutively. A heat wave reduced the number. From a previously prepared Table of random grouping, the animals were divided into two groups. The first group was allowed to survive till 2–7 days and then the lungs were perfused. The other group was allowed to survive till 10–11 weeks and then the lungs were similarly perfused.

Lung Perfusion.

After a light blow on the head the lungs of the guinea-pigs were perfused with Tyrode as described by Feldberg and Kellaway (1937). After the blood was washed out the rate of flow was reduced to 1 ml. per 100 gm. of animal per minute. The rate of flow of the perfusion in the young group was thus 0·5–1·0 ml. per minute. The rate of flow in the perfusion of the older group was similarly 3·9–5·35 ml. per minute, but in one instance where the pig weighed 590 gm. the rate of flow was 1·05 ml./min. This is explained later.

Trypsin (Difco, 20 mg./ml. in Tyrode) was injected into the pulmonary cannula after the first sample of perfusate (S1) was collected and the flow interrupted for one minute. The amount of trypsin injected was 5 mg. per 100 gm. animal. After the flow was re-established the fluid issuing from the lung was collected for 40 minutes (S2). The ventilation rate was 11 per minute and the volume of air used for ventilation increased from the time

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of injection of trypsin to keep the lung equally fully distended so that as much as possible of the fluid in the tissue spaces was released. It thus was found that the volume passed into the pump was regularly increased, commencing at 25–50 ml. per stroke at the time of the trypsin injection and increasing to 45–100 ml. per stroke at the conclusion. After cutting the lungs down, the lungs were drained for 20 minutes. The perfusion (S1, S2) and drainage (D) fluids collected were boiled immediately after collection and the whole lungs were extracted with Tyrode and boiled after being squeezed tightly between filter papers and weighed.

Histamine Assay.

Histamine was assayed as histamine acid phosphate on the isolated jejunum of the guinea-pig suspended in a bath of Tyrode. Rocha e Silva (1940) has already shown that histamine is liberated by trypsin. The response of the gut to trypsin is readily distinguishable from that to histamine (Trethewie, 1942).

EXPERIMENTAL.

Output of histamine from lungs of recently-born pigs.

In spite of the fact that the amount of trypsin injected was proportional to the weight of the pigs and thus the actual amount injected into new-born pigs was quite small, the output of histamine was not negligible. When this is related to the weight of the pig it becomes relatively large. In six instances where the lungs of pigs 2 to 7 days old were perfused and the weight of the pigs ranged from 50–101 gm., the amount of histamine liberated during 40 minutes' perfusion was, including the drainage fluid, 2·4–10·3μg. (av. 6·8 μg.) (Table 1). The lungs after perfusion contained histamine varying in concentration from 94 to 280 μg./gm. (av. 159 μg.). The output of histamine estimated as μg./unit gm. weight of animal varied from 0·02 to 0·21 μg./gm. with an average of 0·10 μg./gm. The details of one experiment are as follows: Pig 8, weighing 70 gm., was sacrificed on the fourth day, the lungs being perfused at a rate of 0·7 ml./min., and following the injection of trypsin (3·5 mg.) during which the pump volume was increased from 40 to 75 ml./stroke, the perfusion fluid collected during 30 minutes after the injection of trypsin (27·6 ml.) contained histamine 1:4·3 mill. The gut failed to respond to trypsin in the dose maximally possible in the test sample. The drainage fluid collected for 30 minutes (1 ml.) contained histamine 1:325,000. The lungs after perfusion contained 100 μg. histamine/gm. Thus 6·4 μg. histamine were liberated in the perfusion and 3·1 μg. in the drainage fluid. The total output of histamine was 9·5 μg., or 0·14 μg./gm.

Output of histamine from lungs of pigs 10–11 weeks old.

The output of histamine from pigs during the third month of life was very much less in proportion. Trypsin was injected in proportion to the weight of the pig so that many times the amount of trypsin was injected into these animals as into those of the first group. The total output of histamine was from 1·9 to 7·1 μg. (av. 4·5 μg.). The concentration of histamine in the lungs after perfusion was 25–48 μg./gm. (av. 34 μg.). The output of histamine expressed in relation to the size of the animal was 0·003–0·018 μg./gm., with an average of 0·010 μg./gm. The findings for one experiment are as follows: Pig 10 was examined when eleven weeks and one day old. The pig weighed 535 gm. and 26·8 mg. of trypsin were injected into the pulmonary artery of the Tyrode-perfused lungs. Perfusion at the rate of 5·35 ml./min. and ventilation at 45 increasing to 100 ml./stroke was undertaken. Much of

TABLE 1.

Liberation of histamine from the perfused guinea-pig lung by trypsin.

Pig No.	Age at perfusion	Weight at perfusion (gm.)	Amount of trypsin (mg.)	Perfusion rate (ml./min.)	Ventilation volume (after trypsin) (ml.)	S2 Concentration histamine (mill.)	S2 Volume (ml.)	D Concentration histamine	D Volume (ml.)	Histamine content lung after (μg./gm.)	Total output histamine (μg.)	Histamine output/ unit weight (μg./gm.)
2	6 days	100	5	1	50–90	1/6	40	1/500,000	0·7	280	8·1	0·08
3	7 days	90	4·5	0·9	40–75	1/7·2	37	1/370,000	0·9	94	7·5	0·08
5	3 days	50	2·5	0·5	35–60	1/2·8	20·1	1/225,000	0·7	160	10·3	0·21
8	4 days	70	3·5	0·7	40–75	1/4·3	27·6	1/325,000	1	100	9·5	0·14
12	4 days	70	3·5	0·7	25–60	1/14	29	1/715,000	0·8	175	3·2	0·05
13	2 days	101	5·05	1	35–45	1/100	41	1/250,000	0·5	143	2·4	0·02
1	10 weeks 5 days	390	19·5	3·9	30–50	1/23	158	1/20 mill.	4	25	7·1	0·018
4	10 weeks 6 days	500	25	5	45–60	1/50	201	1/3·4 mill.	2·9	33	4·9	0·010
10	11 weeks 1 day	535	26·8	5·35	45–100	1/55	210	1/4 mill.	1·2	48	4·1	0·008
11	11 weeks	590	29·5	1·05	30–80	1/25	42	1/12·5 mill.	2·9	29	1·9	0·003

this volume was necessarily taken up in compression in the apparatus volume. The perfusion sample contained histamine 1: 55 mill. (3·8 μg. in a volume of 210 ml.), and the drainage fluid contained histamine 1: 4 mill. (0·3 μg. in a volume of 1·2 ml.). The lungs after perfusion contained 48 μg. histamine/gm. The total output of histamine was thus 4·1 μg. which, expressed in relation to the size of the animal, gives 0·008 μg./gm.

The details in all these experiments are recorded in Table 1. The average weight of the lungs of the older animals was 3·70 gm. (variation 3·2–4·4 gm.) and of the younger 1·34 gm. (variation 0·9–1·8 gm.). The average of the ratio weight of the lungs to weight of animal for the older pigs was 0·0074 (variation 0·0069–0·0082) and the ratio for the younger 0·0179 (variation 0·009–0·027). Thus the output of histamine per gram lung in the case of the younger animals becomes reduced to about four times that for the older animals when estimated on this basis.

In experiment 11 the rate of flow of the Tyrode was reduced to that of the order of the perfusion for the younger animals but still the output of histamine was far less than in the case of that group: in fact, the output was even less than in the other members of its own group. If one considers the output of histamine, namely 0·10 μg./gm. and 0·01 μg./gm., on the basis of weight of the animal, the difference in output in the case of the ten animals is statistically significant. This statement requires the qualification that in one instance of the older-animal group the rate of perfusion was at the rate of the younger group. The reason for making such a comparison on a weight basis is explained in the next section.

Toxicity of Histamine.

The lethal dose of histamine, given subcutaneously, was estimated for animals of the same size as the 10–11 week group and it was found to be 4·8 ± 0·5 mg./kg. (20 animals, probits[2]). Similarly, new-born animals from 2–7 days old were estimated for the $l.d._{50}$, each group containing a corresponding age variance. The $l.d._{50}$ was 3·9 ± 0·4 mg./kg. (24 animals).

Now it is evident that there is no greater capacity of the new-born animals to withstand toxic doses of histamine calculated on a weight basis than those at 10–11 weeks: in fact, if anything the younger animals are more susceptible to injected histamine. If then an injury involving the lung produces a generalized output of histamine the younger animals are less able to withstand the injury so caused. On the other hand if the injury remains localized involving only a limited area of tissue spaces the effect might be a good one for the organism as a local mechanism.

DISCUSSION.

In epidemiological investigations it is recognized that infectious disease seems to affect young age groups from about four to ten years more lightly than the young adult age group (15–35 years). Small infants also may suffer severely. In this paper we have shown that when trypsin, a substance which liberates histamine, is injected into the isolated perfused lungs of guinea-pigs in a fixed concentration and in an amount proportional to the weight of the animal, there is a far greater ouput of histamine per unit weight of animal (some eight or ten times) from the lungs of those animals whose lungs are

[2] These estimates were made by Mr. James through the courtesy of Mr. Cornish, Statistician. C.S.I.R.

perfused within one week of birth compared with those of part-grown animals of the age of ten to eleven weeks. It is probably fair to compare the latter age group with the young age group of humans (4–10 years) since pigs are usually not used for breeding till the age of 17–26 weeks is reached (Worden, 1947), though such a comparison is necessarily only an approximation.

If we can accept that the liberation of histamine is an index of the ease of liberation of other pharmacologically active substances which may be released by infection, this difference in ease of release of histamine may be the basis for the different responsiveness of the young to the infant age group regarding infections. This aspect was commented on in a previous paper (Trethewie and Day, 1948) where it was found that repeated injuries influenced the ease of response of the lung in liberating histamine to another injurious agent. The basis of this increased reactiveness has not been determined in the age groups considered in this paper. It may be a genetically conditioned response to ageing, or endocrine factors may be involved. On the other hand it may be due to the high histamine content of the lungs of the new-born already noted (Trethewie, 1947), though if so we have still not explained why the histamine content is so high at this time. Whatever the explanation, the difference is certainly striking.

SUMMARY.

The output of histamine from the perfused lungs of guinea-pigs after the injection of trypsin is shown to be many times greater in proportion to the weight of the animal in the case of new-born pigs compared with pigs ten or eleven weeks old.

The possible significance of this finding is discussed.

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INFLUENZA VIRUS—ANTIBODY UNION

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In the course of an investigation of material from a recent influenza epidemic at Ocean Island several strains of influenza A virus were isolated in the O phase (Burnet and Bull, 1943). Amniotic fluid or lung emulsions from embryos infected with garglings agglutinated guinea-pig or human cells to relatively high titres but had no action on fowl cells. In agreement with general experience D phase virus capable of agglutinating fowl cells appeared on further chick embryo passage. Serological investigations with ferret antisera showed that the two phases were antigenically indistinguishable.

In the course of this serological work a mixture of O and D virus was used in haemagglutinin-inhibition titrations of ferret antisera with both fowl and guinea-pig cells. The unexpected result was obtained that in the test with fowl cells the mixture of O and D virus gave the same titre as would be given if no O virus were present. Further investigation of this result has led to the view that influenza virus combines preferentially with its antibody in the presence of red cells which it agglutinates. This paper will describe the experiments on which this conclusion is based.

MATERIALS AND METHODS.

Virus Strains.

Ocean Island virus—O phase of influenza A virus isolated by amniotic inoculation in 1948; this gave poor or no agglutination with fowl cells and high-titre agglutination with guinea-pig cells, i.e. low F/G ratio. D phase obtained by allantoic passage of O form; this gave a high F/G ratio.

MEL—Melbourne strain of influenza A isolated in 1935.

Treated MEL—Allantoic fluid of strain MEL heated at 56° C. for 30 minutes in citrate-borate buffer pH 8·5 (Stone, 1949).

BEL—Influenza A strain isolated during Victorian epidemic, 1942.

LEE—Standard influenza B strain.

MIL B—Influenza B strain isolated in Melbourne, 1945.

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Sera.

Ferret sera against the O and D forms of Ocean Island virus and against MEL virus were used. They were taken from ferrets 10–12 days after intranasal inoculation of infected egg material.

Human sera were taken from patients convalescent from influenza infection or following vaccination with the virus.

Haemagglutinin Titrations.

Serial dilutions of antiserum (0·25 ml.) were mixed with an equal volume of 1 p.c. red-cell suspension and a third volume containing 5 agglutinating doses (A.D.) of virus dilution added. The cells were allowed to settle at room temperature and readings were made by observing the pattern of sedimented cells as described by Burnet (1942). Partial, one plus (+) agglutination was taken as the end-point and the titres are expressed as the reciprocals of the initial dilution of serum present at the end-point.

RDE.

The receptor-destroying-enzyme of *V. cholerae* (RDE) was prepared as described by Burnet and Stone (1947).

Periodated Cells.

Packed red cells were treated with an equal volume of M/1,500 potassium periodate for 10 minutes at room temperature. The cells were then washed in saline containing M/100 glycerol to neutralize excess periodate.

PVR Cells.

Virus-coated red cells were prepared by the method of Fazekas de St.Groth (1949), involving the successive treatment of cells with periodate, virus and RDE.

EXPERIMENTS.

The experiment which led to the present investigations was as follows: Ocean Island virus in the O and D forms was titrated with ferret antiserum to the D virus using fowl and guinea-pig cells. As antigens, 5 A.D. of D and 5 A.D. of D virus + 20 A.D. of O virus were used. The titres of inhibition obtained are shown in Table 1.

TABLE 1.

Haemagglutination-inhibition titres of mixtures of O and D viruses with fowl and guinea-pig cells.

Cells	Antigen	
	D virus	O + D virus
Fowl	640	640
Guinea-pig	640	80

It is evident that the mixture of O and D virus behaved as if 25 A.D. virus were present when guinea-pig cells were used but as if only 5 A.D. of virus were present when fowl cells were used. The tentative conclusion was drawn that O virus combined with antibody only in

the presence of the guinea-pig cells, i.e. the cells which it agglutinated. In order to see whether this phenomenon applied to the haemagglutination reaction generally, an experiment was carried out which made use of red cells treated by the receptor-destroying enzyme of *V. cholerae* (RDE).

Suitable treatment of red cells with RDE renders them inagglutinable by influenza viruses (Burnet, McCrea and Stone, 1946). When different concentrations of this enzyme are used to treat red cells it is found that they become inagglutinable by viruses in a definite order, corresponding to the "receptor gradient" (Stone, 1947).

Human group O red cells were therefore treated with varying concentrations of RDE at 37° C. for 30 minutes, followed by citrate to stop further action of RDE, in order to prepare red cells which could be agglutinated by BEL virus but not by MEL. It was found that a final concentration of 1/160 RDE produced this effect. It was now possible to carry out an analogous experiment to that shown in Table 1 by using a human serum which gave the same titre of inhibition with BEL and MEL viruses; normal human cells and cells treated with RDE 1/160 (these would be agglutinated by BEL but not by MEL); and 5 A.D. of BEL virus instead of D (Table 1) and 5 A.D. of BEL + 20 A.D. of MEL viruses (the latter in place of O) as antigens. The results of these titrations are shown in Table 2.

TABLE 2.

Haemagglutination-inhibition titres of MEL and BEL viruses with normal red cells and cells rendered inagglutinable by MEL following treatment with RDE.

Cells	Antigen	
	BEL	BEL + MEL
RDE-treated (inagglutinable by MEL)	240	240
Normal human cells	140	40

From this Table it can be seen that precisely similar results to those shown in Table 1 were found. In the present experiment the MEL virus combined with antibody in the presence of normal human cells but not in the presence of cells rendered inagglutinable by MEL following treatment with RDE. This type of result has been repeatedly confirmed using these strains along with different antiserum and also strains MIL and LEE of influenza B. Control experiments have shown that normal or RDE-treated red cells do not absorb antibody from serum.

Experiments were also carried out to show the presence of unneutralized virus in the supernatant fluids of titrations in which RDE-treated cells were used. Human red cells were treated with 1/40 RDE for half an hour at 37° C. when it was found that they were no longer agglutinated by 40 A.D. of LEE but were still agglutinated by 5 A.D. of MIL virus. A titration was then carried out as described before using a human serum which neutralized both viruses to the same titre, and 5 A.D. of MIL or 5 A.D. of MIL + 40 A.D. of LEE. After the cells had settled readings were taken, the tubes were lightly centrifuged and the supernatant fluids removed. These were tested for agglutinins for normal human cells and cells treated with RDE; any unneutralized LEE virus would agglutinate normal cells but not RDE-treated cells. The results of this experiment are shown in Table 3 where the readings are set out in detail; the figures in brackets represent the titres of unneutralized LEE virus found in the supernatant fluids.

TABLE 3.

Haemagglutination-inhibition titration with LEE and MEL viruses and with normal and RDE-treated red cells.

Cells	Antigen (A.D.)	Dilution of Serum 20	40	80	160	320	640	1,280	Titres
Normal	MIL (5)	—	—	—	—	tr	++	++	480
RDE-treated	do.	—	—	—	—	—	—	+	1,280
Normal	MIL + LEE (5) (40)	— (<6)	— (<6)	— (<6)	++ (<6)	++ (<6)	++ (<6)	++ (<6)	120
RDE-treated	do.	— (<6)	— (<6)	— (<6)	— (<6)	— (<6)	± (10)	±± (18)	1,000

The signs indicate the degree of agglutination of the red cells.
The figures in brackets indicate the haemagglutinin titres of the supernatant fluids in the corresponding tubes.

It can be seen that with MIL and LEE as antigen and normal red cells, any virus which is not neutralized by antibody is adsorbed on to the red cells. When RDE-treated red cells are used excess LEE virus remains in the supernatant fluid and was detected in the tubes on the right-hand side of the Table. In the tubes on the left of the Table the excess antiserum has neutralized the LEE virus. Hence it appears that in the titration with normal cells the antiserum was shared by the MIL and LEE viruses. When RDE-treated cells were used the antiserum combined preferentially with the MIL virus and only excess antiserum was free to combine with the LEE virus.

Inspection of Table 3 shows that less virus was detected in the supernatant fluids than might be expected and the same finding was noted in a further experiment with BEL and MEL viruses. This may be accounted for in part by the results of control experiments which showed that red cells similarly treated with RDE can still absorb some virus although no agglutination results.

Complement Fixation.

Experiments were carried out to see whether influenza virus adsorbed on to red cells functioned as a more effective antigen in fixing complement with antiserum. A red-cell eluate of MEL virus was used along with ferret antiserum. Dilutions of virus were mixed with 3 50 p.c. haemolytic doses of complement and a constant quantity of antiserum added. Parallel titrations were carried out with the addition to the virus of red cells and red cells treated with RDE. After incubating the mixtures at 37° C. for one hour the tubes were lightly centrifuged and the excess complement remaining in the supernatants was titrated. No difference was noted in titrations carried out in the presence of either type of red cells. In an attempt to ensure that virus remained attached to the red cells the experiments were repeated with virus adsorbed to red cells treated with M/1,500 potassium periodate and also to PVR cells (Fazekas de St.Groth, 1949). The same results were found, however. Thus it was shown that virus adsorbed on to red cells did not function as a more effective antigen in the complement fixation reaction.

DISCUSSION.

The mechanism of neutralization of virus by antiserum *in vivo* is not yet clear and suggestions have been made that the host cells participate in the process (e.g. Sabin, 1935). In the present experiments it was found that in haemagglutination-inhibition titrations with a mixture of two viruses, antiserum was shared by the viruses. When however, the experiment was carried out with red cells which were agglutinated by one virus but not by the second, antiserum was preferentially absorbed by the virus which agglutinated the cells. Even when eight times as much of the second virus was present its influence on the haemagglutination titration could not be detected.

According to Burnet, Keogh and Lush (1937) the combination of influenza virus and antibody is a reversible adsorption which may be represented as:

$$V + A \leftrightharpoons VA.$$

The haemagglutinin reaction is a result of adsorption of virus to the red-cell surface and the evidence strongly favours the view that quite apart from the enzymic elution of virus the reaction is a reversible one.

$$V + C \rightleftharpoons VC \text{ or } CVC \text{ (agglutination)}.$$

When the three reagents are mixed a third component of the system AVC becomes possible. The conventional symbols AV and AVC are not, of course, intended to correspond to specific compounds. A virus particle is a relatively large object with a surface bearing probably a considerable number of the atomic groupings responsible for union on the one hand with antibody and on the other with cell receptor.

Under the particular circumstances of the present experiments we have two viruses in the system, V_1 which will attach to either type of red cell and V_2 which will be significantly attached only to one type. The possible components then are:

1. With normal cells:

   $AV_1C \quad AV_2C \quad V_1A \quad V_2A \quad V_1C \quad V_2C;$

2. With the cell susceptible to V_1 only:

   $AV_1C \quad V_1A \quad V_2A \quad V_1C.$

If we adopt this formulation of the reactions concerned the difference between the results observed with mixed viruses (a) on normal cells and (b) on cells to which only one component V_1 is significantly attached must depend on the greater stability of the complex AV_1C than AV_2. In other words, while virus is attached to the cell surface it has either a greater affinity for antibody than has free virus or the temporary stabilization of the virus on the surface allows a firmer union of antibody to virus. It might be suggested that in reversible

reactions such as we are considering the dissociation of any given V–A complex is a result of thermal agitation and that molecular impacts of the necessary energy and orientation are less likely to occur when the system virtually forms part of the solid surface of the red cell. The difference will not of course be absolute and in the presence of an excess of antiserum all virus may be removed from the cell surface. Elford and Dawson (1949) have shown by electron microscope studies that homologous antiserum can remove virus which has been adsorbed on red cells. This in itself is almost decisive proof of the reversibility of the CV union. As each virus particle dissociates from the surface the aspect of the particle previously shielded by contact with cell receptor can adsorb antibody and if the antiserum concentration is high enough the virus particle will remain fully "blanketed" with antibody and unable to attach to cell receptors.

SUMMARY.

Influenza virus in the O form does not bind antibody in haemagglutination-inhibition titrations with fowl cells but does so when guinea-pig cells are used. This phenomenon was found to apply to other strains of influenza viruses A and B which did not bind antibody except in the presence of red cells which they agglutinated.

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SOME OBSERVATIONS ON THE SECRETION OF ACID

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It has been suggested by Davenport (1940a) that the elaboration of acid by the gastric mucosa is mediated by carbonic anhydrase which occurs there in high concentration. Feldberg, Keilin and Mann (1940) in work on cats, have shown that following the venous injection of sulphanilamide (1 gm./kg.) in the two instances where this was done, there was a greater output of acid from the stomach than was the case with two cats used as controls; in both the acid stimulus was histamine (0·5 mg./kg. subcutaneously). We have confirmed these findings in man but a criticism can be applied if this is regarded as disproving Davenport's contention.

If carbonic anhydrase were to have any rôle in the secretion of acid, it seemed worth-while to determine if there is any difference in the carbonic anhydrase level in patients with and without allergy, for it has been shown (Trethewie, 1947) that achlorhydrics are more readily sensitized to one particular sensitizing agent and this was found to be statistically significant. This might indicate an essential tissue difference in allergic subjects. There was no evident correlation between the carbonic anhydrase content of the blood and the degree of acidity elaborated in response to a gastric test meal nor was there a reduced carbonic anhydrase content of the blood of allergic subjects. It was because of these findings that the investigations concerning sulphanilamide were carried out.

METHOD.

Method for the Determination of Carbonic Anhydrase Activity in Human Blood.

Roughton and Booth (1946) have developed a technique for the estimation of carbonic anhydrase activity. They estimate the time required for a quantity of water saturated with CO_2 to reduce the pH of standard Barbitone buffer (pH 7·95) to pH 6·3. The change is gauged by means of bromothymol blue indicator. This procedure is again carried out in the presence of diluted lysed red cells. The speeding up of the time in seconds obtained by the second procedure is taken as an index of enzyme activity.

We have used stoppered weighing bottles throughout and 1 ml. of diluted osmotically lysed human red cells (10, 30, 50, 100, or 1,000 parts per mil. (p.p.m. water), 3 ml. of Barbitone buffer, 1·3 ml. of distilled water and 3 drops of bromothymol blue (0·04 p.c.) were placed

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in the weighing bottle in this order. The bottle was stoppered and placed in ice-water for at least fifteen minutes. The saturated CO_2 was prepared by bubbling CO_2 continuously through distilled water in a wide tube kept in ice and water which thus had an atmosphere of CO_2 over it. 5 ml. of this were taken up in a syringe (kept in ice-water) and carefully squirted into the weighing bottle, taking care to avoid bubbling. The stop-watch was clicked simultaneously. A blank control was used with distilled water in place of the 1 ml. of diluted blood.

Two Barbitone buffers were used. The first buffer (I) was prepared as 0·03 M Barbitone (di-ethyl barbituric acid) + 0·02 M sodium Barbitone, pH 8·3. In later experiments the amount of Barbitone was reduced and buffer II was 0·02 M Barbitone and 0·02 M sodium Barbitone with a pH = 8·4.

The Effect of Sulphadiazine in vitro *on the Carbonic Anhydrase Activity of Human Blood.*

Using the technique described above sulphadiazine (which has the sulphonamide group substituted) was examined for its action in inhibiting carbonic anhydrase enzyme activity. Barbitone buffer II (pH 8·4) and a 30 p.p.m. dilution of normal washed human red cells in water were used. In some of the tests 1·3 ml. of sulphanilamide 1/5,000 was added in place of the usual 1·3 ml. of distilled water, while in others 1/5,000 sulphadiazine was used. The final concentration thus attained in the liquid (10·3 ml.) was 24·2 p.p.m. of the respective drugs. Tests using 30 p.p.m. of blood alone and also blanks were set up. The weighing bottles were randomized, the person performing the test not knowing the nature of the solution tested.

Test Meals.

Test meals were performed at 9 a.m. or 10 a.m., no food having been taken overnight and in some instances water was drunk at 11 p.m., 3 a.m. and 7 a.m. equivalent to that drunk with tablets. The fasting contents and contents at six intervals for the remaining hour following the giving of alcohol were tested for gastric HCl and the total acidity. Each subject later was given sulphanilamide 2 gm. at 7 p.m., then 1 gm. at 11 p.m., 3 a.m. and 7 a.m., very little fluid being taken at 7 a.m. and less than half a cupful at 3 a.m. The blood sulphanilamide was measured at the time of the alcohol meal which followed. These tests were carried out under the direction of Miss Mavis Freeman, Biochemist to the Institute. In some instances the sulphanilamide content of the gastric juice was estimated.

Allergy Patients.

Investigations were carried out on patients suffering from hay fever, allergic rhinitis, asthma and urticaria.

EXPERIMENTAL.

Carbonic Anhydrase in Allergy:

(a) Response of Blood (10 p. Lysed Red Cells p.m.) in Changing pH Veronal Buffer I from 8·3 to 6·3.

The activity of the blood is recorded as the speeding up in seconds of the reaction. In Table 1 are shown the times for the blank control and for the bloods from allergy patients and controls. It is noted that on the whole the carbonic anhydrase activity of the control series is a little greater than that of the allergy series, the speeding up of the reaction being 0·6 sec. with allergy patients, and 3·4 sec. with the control patients on the average. This is not statistically significant and it was considered advisable to use a higher concentration of

enzyme to obtain more conclusive figures, though this might, by increasing the concentration of enzyme, move the times to a less sensitive region making small differences in enzyme activity less obvious.

TABLE 1.

Carbonic anhydrase activity of the blood of allergy and control patients as assessed by the speeding up of the reaction of CO_2 in reducing the pH of buffer I to 6·3 when 10 p. lysed red cells p.m. was added to the system.

Allergy patients			Control patients		
Patient No.	Blank (sec.)	Carbonic anhydrase activity (sec.)	Patient No.	Blank (sec.)	Carbonic anhydrase activity (sec.)
1	77	3·6	1	77	1·4
2	77	4·4	2	77	3·1
3	77	0·3	5	75·4	3·8
8	76·5	2·5	6	75·4	4·8
9	76·5	1·3	7	75·7	2·9
10	74·2	7·0	8	75·7	4·6
11	75·7	—2·6			
12	75·7	—5·3			
13	75·7	—8·1			
14	75·7	2·3			
		Av. 0·6			Av. 3·4

(b) Response of Blood (30 and 50 p. Lysed Red Cells p.m.) in Changing pH Veronal Buffer II from 8·4 to 6·3.

The buffer used in this instance and for all later experiments was Number II (pH 8·4) and the findings are shown in Table 2. It is noted that there is no significant difference in the times, e.g. with 30 p. lysed red cells p.m. the average speeding up of the time was 5·9 sec. for allergy patients and 5·8 sec. for the controls. Thus there is no difference in the enzyme content of blood in these series between allergy patients and controls and from the findings it does not seem worth-while to increase the series. When 50 p. cells p.m. were used and 100 p.p.m. (Table 2) it is also noted there was no significant difference in the times.

It is evident that we have not been able to show any difference in the carbonic anhydrase activity of the blood of allergy and control patients.

Gastric Acidity in Relation to Carbonic Anhydrase:

(a) Allergy Patients.

It is noted (Table 3) that there is considerable variation in the gastric acidity to alcohol meal in these patients. It varies from achlorhydria to a free acid (HCl maximum) 96° (100 ml. content equivalent to 96 ml. N/10 NaOH). The total HCl is recorded in Table 3 as the sum of the seven sample levels. The "unit" is measured by the sum of the volume of the fasting content (ml.) multiplied by the acidity and the volume (ml.) of the residual content multiplied by its acidity. This was recorded in an attempt to overcome some of the difficulty inherent in not knowing the volume of acid secreted. In eight instances out of fourteen the gastric acidity barely rose over 20°. The other maxima were 25°, 34°, 43°, 49°, 50° and 50°. It is noted (Table 3) that there is no evident correlation between the degree of acidity and the activity of the carbonic anhydrase with either 10, 30 or 50 p. lysed red cells p.m. This does not suggest that carbonic anhydrase might be related to the degree of output

TABLE 2.

Carbonic anhydrase activity of the blood of allergy and control patients as assessed by the speeding up of the reaction of CO_2 in reducing the pH of buffer II to 6·3 when 30, 50 and 100 p. lysed red cells p.m. was added to the system.

30 p.p.m.						50 p.p.m.						100 p.p.m.					
Allergy patients			Control patients			Allergy patients			Control patients			Allergy patients			Control patients		
Patient No.	Blank (sec.)	Carbonic anhydrase activity (sec.)	Patient No.	Blank (sec.)	Carbonic Anhydrase activity (sec.)	Patient No.	Blank (sec.)	Carbonic anhydrase activity (sec.)	Patient No.	Blank (sec.)	Carbonic Anhydrase activity (sec.)	Patient No.	Blank (sec.)	Carbonic anhydrase activity (sec.)	Patient No.	Blank (sec.)	Carbonic Anhydrase activity (sec.)
20	72·1	3·4	16	72·1	1·1	20	72·1	10·3	16	72·1	9·5	18	72·7	18·8	11	72·7	20·3
21	72·1	3·6	17	72·1	5·7	21	72·1	13·5	17	72·1	13·0	19	72·7	16·6	12	72·7	16·5
22	72·1	7·4	18	71·8	4·6	22	72·1	15·0	18	71·8	12·6				13	72·7	17·3
23	72·1	9·3	19	71·8	3·5	23	72·1	12·8	19	71·8	12·7						
			20	72·3	10·3				20	72·3	15·2						
			21	72·3	9·7				21	72·3	14·6						
	Av.	5·9		Av.	5·8		Av.	12·9		Av.	12·9		Av.	17·7		Av.	18

TABLE 3.

Relationship of gastric acidity to the carbonic anhydrase activity of the blood as assessed by the speeding up of the reaction $H_2O + CO_2 \rightarrow H+ + HCO_3-$.

Allergy patients								Control patients							
	"Acidity"			Speeding up of reaction (sec.) (carbonic anhydrase activity)					"Acidity"			Speeding up of reaction (sec.) (carbonic anhydrase activity)		
				p.p.m. cells								p.p.m. cells		
Patient No.	"Unit" HCl	Max. HCl	Total HCl	10 (Buffer 1)	30 (Buffer 2)	50 (Buffer 2)	100 (Buffer 2)	Patient No.	"Unit" HCl	Max. HCl	Total HCl	30 (Buffer 2)	50 (Buffer 2)	100 (Buffer 2)
P8	78	23°	50	2·5				S2	525	18°	62	5·4	8·2	
11	288	16°	55	—2·6				3	120	10°	15	8	11·2	
14	262	20°	100	2·3				6	0	0	0	6	8·6	
19	134	96°	523				16·6	8	5,625	73°	376	6·9	10·2	
22	175	28°	93		7·4	15		1	3,640	52°	210			
21	1,350	50°	265		3·6	13·5		4	0	5°	15			
S9	144	34°	153		4·1	8·7		5	0	0	0			
P25	639	17°	54					7	0	5°	5			
4	1,260	50°	231											
16	480	25°	123											
15	0	0	0											
24	654	21°	126											
3	919	43°	162											
6	1,320	49°	111											

of acid. It is to be remembered that the enzyme activity we measure is that in the blood and not in the gastric mucosa.

(b) Control.

Of eight test meals of control subjects two showed achlorhydria, in four the gastric acidity did not rise above 20° and the other maxima were 52° and 73°. The carbonic anhydrase activity of these was also determined and is recorded in Table 3. It is again noted that there is no strict correlation between the carbonic anhydrase content of blood and the acid output, e.g. subject S3 gave a carbonic anhydrase "activity" of 8 sec. (30 p. lysed red cells p.m.) with a maximum HCl acidity of 10°, while subject S8 gave an enzyme "activity" of 6·9 sec. (30 p.p.m.) against a maximum HCl acidity of 73°.

Inhibition of Carbonic Anhydrase:

In confirmation of the work of Mann and Keilin (1940) we have been able to show (Table 4) that sulphanilamide in a concentration of 24·2 p.p.m. causes inhibition of carbonic anhydrase enzyme activity as indicated by the reduction of the time required to lower the pH of a veronal buffer from 8·4 to 6·3 when CO_2 is added. We have also shown (Table 4) that sulphadiazine is without activity. This is in agreement with the findings of Mann and Keilin who indicated that it was necessary to keep the hydrogens in the SO_2NH_2 group intact if the enzyme inhibition were to occur. Sulphadiazine has this grouping altered.

TABLE 4.

Effect on speed of reaction $CO_2 + H_2O \rightarrow H+ + HCO_3-$ to fix pH 6·3 for buffer II of sulphanilamide and sulphadiazine when 30 p. lysed cells p.m. is used.

Blank (sec.)	Blood alone 30 p. cells p.m. (sec.)	Sulphanilamide 24·2 p.p.m. (sec.)	Sulphadiazine 24·2 p.p.m (sec.)
68·1	64·3	68·8	66·2
67·4	61·9	67·2	62·0
64·2	59·0	67·6	61·8
63·9	62·8	68·4	61·6
64·1	58·8	67·8	58·6
72·4	58·0	60·8	60·5
65·1	61·0		
68·8	61·1		
68·1	60·3		
67·0			
65·5			
65·2			
68·3			
67·5			
Av. 66·9	60·8	66·8	61·8
Activity (sec.)	6·1	0·1	5·1

The average time of the blank controls for the reduction of buffer II to pH 6·3 was 66·9 sec. and the average time when 30 p. lysed red cells p.m. was used was 60·8 sec. The average time for the samples containing the drugs 24·2 p.p.m. was, however, 66·8 sec. for sulphanilamide, and 61·8 sec. for the blood containing sulphadiazine. Thus we see that although the 30 p.p.m. blood has a carbonic anhydrase activity sufficient to speed the reaction

up by 6·1 sec., when 24·2 p.p.m. of sulphanilamide is present this activity is nullified. The story with sulphadiazine is different, however, even with a concentration of 24·2 p.p.m. the carbonic anhydrase activity is sufficient to increase the rate of reaction by 5·1 sec., only 1·0 sec. slower than when no drug was present and within the limits of experimental error. Thus sulphanilamide has the effect, in the concentration of drug and enzyme used, of antagonising carbonic anhydrase *in vitro* in blood whereas sulphadiazine which has a substituted sulphonamide grouping has no antagonistic action in these concentrations.

Investigation of The Role of Carbonic Anhydrase in the Elaboration of Gastric Acid:

Davenport (1940 a, b) has suggested that carbonic anhydrase is concerned in the elaboration of gastric acid. The antagonism of sulphanilamide to carbonic anhydrase presents a convenient approach to this problem and indeed has been used by Feldberg, Keilin and Mann (1940) in cats. It was found that following the injection of large amounts of sulphanilamide there was no inhibition of acid secretion. Our experiments were carried out on human subjects (medical students). Two alcohol test meals were given to each student. The second was performed after a schedule of sulphanilamide in some students and sulphadiazine in one. The level of the drugs present in the blood was checked at the time of the second test meal and it reached a level of 4·2–7·2 mg./cent. with sulphanilamide and 13 mg./cent. with sulphadiazine.

TABLE 5.

Total free HCl (gastric juice) in subjects before and after ingestion of sulphanilamide.

	Total free HCl*			
No.	Control	Sulphanilamide	Water control	Increased output with sulphanilamide
S1	201	215		14
2	61	230		169
3	15	82		67
4	15	0		—15
5	0	0		0
6	0	131		131
7	5	63		58
9	153	232	30	140
10		0	0	0
11		220	197	23
Av.	56	117	76	59

* Ml. NaOH N/10 neutralizing 100 ml. gastric content (total of all samples).

In the cases of students receiving sulphanilamide the "total" acidity varied from 0 to 232 (average 117) while in the controls 0 to 201 was the variation (average 59). These findings are recorded in Table 5. Thus there was a greater output of acid in subjects receiving sulphanilamide. This is contrary to expectations if carbonic anhydrase is concerned in the elaboration of gastric acid unless a reverse phenomenon occurs. The student receiving sulphadiazine showed no appreciable change in total gastric acidity, a rise of 3 p.c. from 378 to 390 being observed.

Statistical analysis gives a standard deviation of 67·4 and a mean difference of 59. This is significant ($p = 0{\cdot}025$).

In eight cases no fluid was taken with the control and in three fluid was taken in the control of the same amount as when the tablets were swallowed at 7 and 11 p.m., 3 and 7 a.m. The average difference in acid output with full water controls was 75 and in the cases without water controls 63. In the one instance where both types of control were applied there was a greater difference in output when compared with the water control.

When the interval between the last dose of sulphanilamide and the test meal was increased to 3 hours, the findings were similar.

Inhibition of Carbonic Anhydrase Activity in High Concentrations (1,000 p.p.m.) of Cells by Sulphanilamide:

Since the amount of carbonic anhydrase inhibited by sulphanilamide in the preceding work was presumably comparatively small, it was thought advisable to show that sulphanilamide also inhibited higher concentrations of enzyme. It was found that concentrations of cells greater than 1,000 p.p.m. were impracticable for the test as they interfered with the colour and it was not possible to distinguish the end-point. Using a 1,000 p.p.m. dilution of normal cells giving a final concentration of 97 parts/mil. system the time taken to change the pH of buffer II from 8·4 to 6·3 was 11·4 sec. The blank control was 71·0 sec. When sulphanilamide was added to the 1,000 p. lysed cells p.m. in order to obtain a concentration of 8·0 mg./cent. (80 p.p.m. of drug) the lysed cell concentration now being 97 parts/mil. system there was almost complete inhibition of the enzyme activity, 67·8 sec. being required for the reaction. Thus the 97 p.p.m. cells have an enzyme activity equivalent to 59·6 sec. and this activity is reduced to 3·2 sec. by 80 p.p.m. sulphanilamide. Thus the drug also inhibits larger quantities of enzyme.

TABLE 6.

Effect of descending concentrations of sulphanilamide on enzyme activity of 97 p. lysed cells p.m. (Buffer II).

Concentration of Sulphanilamide	Reaction time (sec.)		Carbonic anhydrase enzyme activity (sec.)
	Blank	97 p. lysed cells p.m.	
	77·9		
Nil		13·2	64·7
80 p.p.m		59·8	18·3
40 p.p.m		58·8	19·3
20 p.p.m.		49·4	28·5
10 p.p.m.		43·3	34·6
5 p.p.m.		38·7	39·2
2·5 p.p.m.		34·7	43·2
1·25 p.p.m.		28·5	49·4

A descending series of concentrations of sulphanilamide was then tested when 1 ml. of 1,000 p. lysed cells p.m. was added to the system (97 p.p.m. system). There was inhibition of most of the enzyme activity by 80 p. sulphonamide p.m. while even 1·25 p. sulphonamide p.m. caused definite inhibition of activity from 64·7 sec. to 49·4 sec. These figures are shown in Table 6, and represent averages of three estimations.

In another experiment sulphanilamide was added to a 10 p.c. dilution of cells to make a concentration of 8·25 mg./cent. and 0·825 mg./cent. with 1,000 p. lysed cells p.m. The former value is a little greater than the average amount of sulphanilamide present in the

plasma of students after the schedule of the drug. Tests were performed on this without further addition of sulphanilamide (the system now containing sulphanilamide 0·8 and 0·08 mg./cent. and the cells 97 p.p.m.). There was still definite inhibition of enzyme activity. Thus the time taken to change the pH of the buffer from pH 8·4 to pH 6·3 was increased from 10·3 sec. in the case of the 97 p. lysed cells p.m. to 37·1 sec. with that containing 8 p. sulphanilamide p.m. system and to 22·9 with that containing 0·8 p. sulphanilamide p.m. system. Thus sulphanilamide has a definite inhibitory action on the carbonic anhydrase enzyme activity of lysed red cells when the lysed red cells are in relatively high concentration.

If the relative effect is similar when the concentration of both constituents is increased then 8,000 p.p.m. sulphanilamide is required to produce noticeable inhibition of enzyme activity in a gastric cell. The concentration in the blood was actually only 42–72 p.p.m. in our subjects. This would suggest that in the gastric cell such a concentration of sulphanilamide would be without effect on carbonic anhydrase. In other words the concentration of carbonic anhydrase present may be too great to be inhibited by the concentration of sulphanilamide present in the body. In the work of Feldberg, Keilin and Mann a concentration of sulphanilamide of 10,000 p.p.m. blood was used in cats. Thus, if cat gastric cells are as sensitive as human red cells and they become concentrated with this dose in the hour, the lack of inhibition of acid output is significant. Cat gastric cells, however, contain five times as much of the anhydrase as do cat red cells.

In a further endeavour to clarify this situation, since we did produce significant effects on acid output as determined by the test meal when only a small concentration of sulphanilamide was present in the blood (and it might well be that the concentration of sulphanilamide in the gastric cells within two hours of the last oral dose is much higher than that in the blood as large amounts of sulphanilamide lay in direct contact with them), we also determined the sulphanilamide content of the gastric juice. In fact the concentration of sulphanilamide in the gastric juice three hours after the ingestion of the last dose of sulphanilamide was from 60–2,200 p.p.m., while two hours after the last dose of sulphanilamide it was 130 p.p.m. in the one instance so tested.

DISCUSSION.

It is evident that there is no difference in the carbonic anhydrase content of the blood of patients suffering from the various allergies described and of apparently healthy subjects. This investigation was carried out in an attempt to determine some fundamental difference biochemically in patients developing allergy from "normal" subjects since it has been shown that sensitivity to mustard gas is more readily developed in achlorhydrics than non-achlorhydrics (Trethewie, 1947). It may be that since there is such a general intense exposure to the antigens which produce the conditions of allergy investigated the subjects were not necessarily more readily sensitized than other individuals, natural variation being sufficient to explain the development of allergy in those patients. It is not unlikely that anyone can become sensitized to any antigen if there is sufficient exposure. Even in the subjects suffering from achlorhydria there was no difference in the carbonic anhydrase content of the blood compared with that in "normals". This may not preclude the likelihood of the gastric mucosa in such subjects being deficient in carbonic anhydrase. However, in the absence of this information it was considered inadvisable to pursue further

the relation of carbonic anhydrase to allergy and to achlorhydria. It seemed more important to investigate the influence of an inhibitor (sulphanilamide) on carbonic anhydrase and gastric acidity in humans.

Mann and Keilin have already shown (1940) that sulphanilamide inhibits carbonic anhydrase. Feldberg, Keilin and Mann (1940) then investigated the effect of sulphanilamide on the output of acid in response to histamine from the stomach of the pithed cat. It was evident that there was no reduction in acid output in these animals. It may be thought that insufficient sulphanilamide might reach the gastric cell to be effective in inhibiting this action. As far as the quantitative analysis has been approximated by us sufficient sulphanilamide might reach the cell to produce this effect if none were lost following injection into the vein during the hour it was allowed to act and if the cat parietal cell contained as much carbonic anhydrase as the human red cell. However, the cat parietal cell contains five times as much carbonic anhydrase as the cat red cell. It would seem that there is some chance that sufficient sulphanilamide would penetrate the parietal cell to produce its effect. Feldberg, Keilin and Mann contended from their work that carbonic anhydrase is not directly concerned in the elaboration of acid. In the two experiments performed by them there was a greater output of acid than in the same number of controls.

On the other hand, it seems that a distinction has not been made between the elaboration of acid in the parietal cell, which could still be mediated by carbonic anhydrase, and the release of acid into the lumen of the stomach which might not be inhibited by sulphanilamide. Admittedly this would require some "combined form" or other mechanism to have the HCl inactive inside the cell, but this is the rule with pharmacologically active chemicals as e.g. histamine. Further the parietal cells do stain intensely with the acid stain. It could still be that carbonic anhydrase is important, as Davenport has suggested, in the formation of HCl and a careful examination of a diagram indicates (Fig. 1) that HCl is indeed only formed inside the cell by this mechanism with intracellular carbonic anhydrase. Chloride ions pass into the protoplasm to make up for the HCO_3 ions which pass out of the protoplasm into the surroundings. Thus, one cannot conceive how sulphanilamide, having admitted it inhibits carbonic anhydrase enzyme activity, can stop the release of HCl to an alcohol test meal if it is already preformed somehow in the protoplasm. In the one experiment where we carried out a second control test meal one month after sulphanilamide administration, there was a very great difference in the output of acid, there being only 30 ml. equivalents of acid released compared with 232 following sulphanilamide and 153 with the first control prior to sulphanilamide. This would not disagree with sulphanilamide inhibiting the formation of HCl in the cell so that a later meal may only allow a small output of acid. This will be further pursued when further subjects are available. This contention would be untenable if in the short period of the test new

formation of acid were more important than that already preformed. One further point requires elaboration. Davenport (1940 b) assessed the carbonic anhydrase content of cells from pieces of mucosa and inferred, owing to the distribution of the parietal cells, that the parietal cells contained a much greater amount of carbonic anhydrase than the remainder. While this is probably true, thoroughly incontrovertible evidence for the presence of carbonic anhydrase inside the parietal cells has not been demonstrated by this technique.

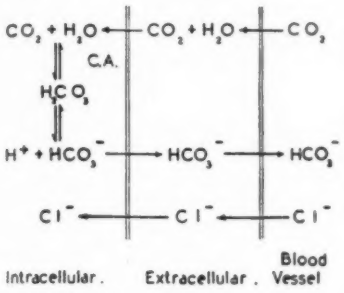

Fig. 1. Schematic diagram of the rôle of carbonic anhydrase in the formation of HCl in the gastric mucosa following Davenport's theory.

The reason for the increase in output of acid following the giving of sulphanilamide seems obscure. Admittedly we have not a very accurate method for determining the acid response to the test meal since we do not know the volume of gastric juice secreted. We have made an attempt to approximate this figure in the first part of the paper by multiplying the volume of the fasting contents by the level of acid and adding to that the volume of the residual content at sixty minutes multiplied by its content of acid. This does not tell us how much acid is actually poured out even at these times because liquid is continually passing the pylorus. However, if the motility of the stomach and water output is unaffected by sulphanilamide, since each subject made his own control, we would be justified in saying that sulphanilamide causes an increase in the output of acid.

On the other hand it may well be that the level of sulphanilamide in the parietal cell is far greater than in the blood because the sulphanilamide is taken orally and passes over the gastric mucosa thereby enabling a higher quantity of sulphanilamide to lie against the gastric cells and, owing to the level of the pressure gradient, one might expect reasonable absorption to occur since it is a simple compound. Estimations made of the level of sulphanilamide in the gastric content three to four hours after the last dose show levels extending up to 220 mg./cent. (2,200 p.p.m.). Presumably the level was greater at the time the tablets were ingested and thus it could well be that the gastric cells may have a higher concentration still within their protoplasm from which slow diffusion only would take place into the blood. If this is so, we may have more than 2,200 p.p.m. sulphanilamide. Now, we have suggested, subject to extrapolation errors, that a concentration of 8,000 p.p.m. sulphanilamide might be required to produce a noticeable inhibition of enzyme activity in the gastric

cell. Having due regard to the possible grossness of the error in such approximations, it would seem that there is at least a likelihood that high levels of sulphanilamide may be present in the gastric cell sufficient to produce effects on carbonic anhydrase. Undoubtedly this would only produce great effects on surface cells and not those in crypts from whence fluid is issued outward to the stomach lumen. Whatever the cause of the increase in acid output following ingestion of sulphanilamide, it would only be expected that a fraction of the usual response of all the cells (i.e. those on the surface) would be influenced by sulphanilamide if such a high concentration as has been suggested were really necessary to influence the acid output. This may be the reason for the fact that the increase in output of acid was approximately 100 per cent. and not a greater multiple as might have been expected were the same mechanism completely inhibited which had been operative previously. On the other hand, if the mechanism of the increase in acid output is not related to carbonic anhydrase, such a high concentration may not be necessary to influence the level of acid released. We can expect the movement of chloride from the cell by inhibition of carbonic anhydrase by a reversal of the mechanism and this conceivably might enter the gastric content but this gives no explanation of the concomitant release of hydrogen ion. However, it seems obscure how sulphanilamide can produce an increase in acid output by inhibition of a carbonic anhydrase mechanism. It is not unlikely that this influence is entirely unrelated to such a method.

SUMMARY.

The carbonic anhydrase activity of human blood has been estimated both in allergy patients and in normal controls but there was no difference in the content of enzyme in the two groups so estimated.

The gastric acidity, as estimated by test meal, of allergy and control patients in these series were comparable.

The giving of sulphanilamide orally in human subjects was followed by an increase in the output of acid to alcohol test meal.

Comparative estimates of the inhibition of carbonic anhydrase by sulphanilamide were used as evidence in discussing the rôle of carbonic anhydrase in acid secretion.

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THE METABOLISM OF ERYTHROCYTES

V. THE INHIBITION OF METHYLENE BLUE RESPIRATION[1]

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In a previous communication (Nossal, 1948) it was shown that, in the presence of methylene blue (M.B.), a number of substances, especially hexoses, could serve as substrates for the respiration of rabbit erythrocytes. The suggestion of earlier workers, that, for hexoses, preliminary phosphorylation occurs prior to oxidation, was confirmed by certain manometric manipulations.

Although the more common inhibitors, such as fluoride and iodo-acetate, have been investigated for this system by earlier workers (Moruzzi, 1936, 1937; Kiese, 1944) no comprehensive range has been reported. In this work, the effects of some twenty-five inhibitors are described, and their site of action (i.e. whether in the initial phosphorylation or in the actual oxidation) discussed. For the purposes of simplicity, it is proposed to call "direct oxidation of glucose" that process which occurs *after* the glucose is phosphorylated but *before* it is split to triose compounds; the term is not intended to imply oxidation of glucose as such.

Methods.

Most of the methods employed are described in the earlier work. Inhibitors were made up in isotonic phosphate-saline, after being neutralised if necessary. All measurements of oxygen uptake were made with standard Warburg equipment at 38° C. M.B. concentration was 0·0033 p.c. throughout, and that of glucose M/100.

Each inhibitor was tested in three ways: Firstly, against lactate, in which case it does not matter whether substrate and inhibitor are pipetted into the main compartment or the side-bulb (S.B.) of the Warburg cups, since lactate is not metabolised by the cells. Secondly, both glucose and inhibitor were placed in the main compartment and the cells preincubated with them for an hour prior to M.B. tipping. Thirdly, glucose was put in the main compartment and the inhibitor in the S.B., the latter being then tipped in with the M.B. after an hour's pre-incubation.

Occasionally, both glucose and inhibitor were tipped from S.B.'s simultaneously with the M.B.

If inhibition of the lactate oxidation occurs, the inhibitor is suggested to affect the oxidative system (haemiglobin reductase, Kiese, 1944) since no preliminary transformations are required. In the case of glucose, rôle in phosphorylation and in oxidation can usually be determined by comparing the severity of inhibition under the two procedures cited above: If

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more pronounced when the inhibitor is present during the preincubation (when phosphorylation of glucose is known to occur) than when the inhibitor is only added subsequently, then it is suggested that its action is on the transformations which glucose must undergo prior to oxidation.

RESULTS.

Iodoacetate and Fluoride.

Inhibition of the reconversion of methaemoglobin to oxyhaemoglobin by these inhibitors has been described by Drabkin (1946) and Kiese (1944). Moruzzi has studied their effect on the M.B. respiration of erythrocytes (1936, 1937).

Results obtained in this work confirm the above observations. Both substances, tested against glucose, inhibit *not* the direct oxidation, but the processes preceding it, as shown by the much stronger inhibition obtained when they are present during preincubation (Tables 1 and 2).

TABLE 1.

Inhibition of M.B. respiration by iodoacetate and fluoride.

Conditions: For fluoride, M.B. in S.B., glucose and inhibitor in main compartment, 1 hour's preincubation. For iodoacetate, all three constituents in S.B.'s, no preincubation. Experimental period: 3 hours.

	Percentage inhibition*	
Molarity of inhibitor (final)	Fluoride	Iodoacetate
10^{-2}	72–78	70–75
5×10^{-3}	69–77	
2×10^{-3}	51–60	
10^{-3}	34–46	34–41
10^{-4}	none	25–28
10^{-5}	none	1–11

*In all cases, percentage inhibition $= \frac{O_2 \text{ uptake of control} - O_2 \text{ uptake of experiment}}{O_2 \text{ uptake of control}} \times 100$ taken over the experimental period.

TABLE 2.

Variation of fluoride and iodoacetate inhibition with experimental conditions.

Inhibitor concentration: 10^{-2} M. Experimental period: 3 hours.

	Percentage inhibition	
	Fluoride	Iodoacetate
Condition 1	58–65	70–75
Condition 2	37–40	35–44
Condition 3	72–78	82–90

Condition 1 = Glucose, M.B. and inhibitor all added after equilibration.
Condition 2 = Glucose preincubated with cells 1 hour, *then* M.B. + inhibitor added.
Condition 3 = Glucose + inhibitor preincubated with cells 1 hour, *then* M.B. added.
for this and all subsequent Tables.

This is supported by the following experiments: six pairs of cups were used. All contained glucose + M.B. in one S.B., and six only contained inhibitor in the other. After equilibration, glucose and M.B. were added in all (zero time), and the inhibitor added to the experimental cups at 0, 5, 15, 25, 35 and 45 minutes. If the *oxidation* were affected, one would expect considerable differences in inhibition over the first hour; this was not found to be the case.

On the other hand, when iodoacetate was added to cells + glucose at different intervals during the preincubation, great variations were found in the subsequent O_2 uptakes when M.B. was added.

TABLE 3.

Effect of exposing cells plus glucose to iodoacetate for varying periods, prior to M.B. addition.

Preincubation: 1 hour.

Experimental period (after tipping of M.B.): 3 hours.

Time of addition of inhibitor from commencement of incubation (minutes)	Percentage inhibition calculated from control
0	90
15	83
30	57
45	40
60	31

Inhibition was progressive for both fluoride and iodoacetate; with 10^{-2} M F–, 100 p.c. inhibition is observed during the third hour after M.B. addition.

It may be noted that Gibson (1948) claims that fluoride has no inhibitory effect on haemiglobin reduction by glucose.

Studies of reversal of iodoacetate by excess cysteine were complicated by the fact that cysteine is (a) autoxidizable, (b) oxidized by erythrocytes or M.B., (c) oxidized more rapidly still by erythrocytes + M.B. and (d) both (b) and (c) are inhibited by iodoacetate.

However, it was possible to carry out experiments which show that up to 50 p.c. reversal of inhibition by 10^{-3} M iodoacetate was effected by 10^{-2} M cysteine.

Altogether, the results indicate that the inhibitions observed can be accounted for by their known action on glycolysis (for fluoride, inhibition of enolase, and for iodoacetate, inhibition of triose phosphate dehydrogenase, both of which will have an effect on the phosphorylation of glucose) without stipulating interference in the direct oxidation.

Phloridzin.

This substance was tested because of its marked effect on glucose resorption in the kidney. As expected, it produced a marked inhibition of glucose oxidation (Table 4). Furthermore, the inhibition was again strongest when the inhibitor was present during the initial preincubation period of the cells with glucose alone. These results afford fairly direct evidence that phloridzin is intimately connected with the hexokinase system. Furthermore, phloridzin had no effect whatsoever on lactate oxidation, suggesting that it does not act merely by affecting the cell permeability.

TABLE 4.

Inhibition of respiration on glucose by phloridzin.

Condition 3 throughout.

Final concentration of phloridzin (M)	Percentage inhibition of O_2 uptake after M.B. addition
10^{-2}	77–87
3×10^{-3}	72–80
10^{-3}	44–63
10^{-4}	9–14
10^{-5}	8–10
10^{-6}	4–12

Copper.

At high concentrations, Cu++ caused extensive cell damage under certain conditions. Glucose, M/100 Cu++ (dissolved by means of a little Na citrate, with its pH adjusted) and cells could be incubated without any apparent harm. But when M.B. was added, evolution of gas and parallel lysis of the cells occurred. Over a three hour period, a 100 p.c. inhibition of O_2 uptake occurred.

For 10^{-3} M Cu++, a great reduction of O_2 uptake set in over the first 1½ hours, and *then* a small *output* was observed. Microscopic examination showed extensive lysis. For 10^{-4} M Cu++, the inhibition of O_2 uptake was less than 20 p.c., the cells showing no lysis but some having serrated edges.

For lactate, a much slighter inhibition (up to 20 p.c. for 10^{-3} M Cu++) was observed.

The effect of Cu++ is difficult to explain: The possibility cannot be excluded that, at pH 7·4, it reacts with the glucose, thereby removing the main oxidizable substrate, and inhibiting respiration. Alternatively, it may be a real inhibitor of haemiglobin reductase. Neither explanation shows why lysis should occur, although the latter must be connected with the presence of Cu++ and methaemoglobin (or Cu++ and M.B.).

Pyruvate.

Sufficiently high concentrations of pyruvate have for long been known to inhibit severely the oxidation of lactate. This effect has already been described in the M.B. respiration of erythrocytes on lactate (Wendel, 1933); in confirmation, 10^{-2} M pyruvate was here found to inhibit oxidation of rather higher concentrations of lactate up to 75 p.c.

It was of interest, therefore, to examine the effect of pyruvate on glucose oxidation, to see how much of the total O_2 uptake with glucose was due to the oxidation of lactate produced glycolytically.

Pyruvate inhibition over 90-minute experimental periods was appreciable, reaching 30 p.c. under certain conditions (Table 5). It can be seen that inhibition was more pronounced in the system in which glucose had been preincubated, suggesting that at least part of the increase of O_2 uptake after preincubation of cells with glucose may be due to formation of lactate.

TABLE 5.

Effect of pyruvate on M.B. respiration.

Pyruvate = 10^{-2} M. Experimental period: 1½ hours.

Preincubation (if any): 1 hour.

Substrate	Concentration (M)	Conditions*	Overall percentage inhibition
Lactate	2×10^{-2}	1	69–75
Glucose	10^{-2}	2	19–30
Glucose	10^{-2}	1	15–17

* See Table 2.

Nicotine.

This alkaloid has been claimed a strong inhibitor of lactate oxidation (Baker *et al.*, 1938). In erythrocytes, however, the effect of this poison is surprisingly small (Table 6). In addition, it is *not* the oxidation of lactate which is most affected, but that of glucose, exactly the opposite from what was found in brain.

TABLE 6.

Effect of nicotine on M.B. respiration.

Both substrates 10^{-2} M; preincubation and experimental periods as in Table 5.

Substrate	Inhibitor concentration (M)	Conditions*	Overall percentage inhibition
Lactate	$1–3 \times 10^{-2}$	1	0–20
Glucose	$1–2 \times 10^{-2}$	2	12–17
Glucose	$1–2 \times 10^{-2}$	3	22–36

* See Table 2.

The inhibition is weaker than in other tissues (ranging from 33 p.c. in testes to 91 p.c. in brain, Baker *et al.*, 1938). The effect of nicotine is again more pronounced when the inhibitor is present during the preincubation of glucose, suggesting sensitivity of the glycolytic rather than the oxidative enzymes.

Quinine.

This suspected inhibitor of hexokinase (Marshall, 1948) was found to be very effective against the oxidation of glucose. Whereas Marshall suggests, however, that lactic dehydrogenase may be another point of action, lactate oxidation by erythrocytes + M.B. was here found to be almost entirely insensitive to quinine. The inhibition was still noticeable with 10^{-3} M quinine (Table 7).

Similarly, *caffeine* also affects the initial transmutation of glucose rather than the direct oxidation, and has no effect whatsoever on lactate oxidation (Table 7).

TABLE 7.

The effect of quinine and caffeine on glucose oxidation.

Glucose: 10^{-2} M; preincubation, 1 hour.

Experimental period after M.B. addition: 1½ hours.

Inhibitor	Concentration (M)	Conditions*	Overall percentage inhibition
Quinine	5×10^{-3}	3	49–61
Quinine	5×10^{-3}	2	22–35
Quinine	$6{\cdot}2 \times 10^{-4}$	3	14–29
Quinine	$6{\cdot}2 \times 10^{-4}$	2	10–13
Caffeine	10^{-2}	3	53–63
Caffeine	10^{-2}	2	33–42

* See Table 2.

Diethyldithiocarbamate.

This substance, a strong inhibitor especially of Cu enzymes, was found to inhibit O_2 uptake with glucose as substrate to the extent of *ca* 15–25 p.c. for concentrations of $3{\cdot}3$–$8{\cdot}3 \times 10^{-3}$ M. Lactate oxidation was again insensitive. In this case, it was not possible to show clearly whether the inhibitor affected glycolysis or oxidation.

Nitrophenols.

The nitrophenols have long been known to stimulate respiration of whole animals. Their only connection with enzyme systems has been a suggested inhibition of phosphorylative enzymes. This is supported for the erythrocyte systems: Both 2:4-dinitrophenol and p-nitrophenol were found to inhibit glucose oxidation, but not oxidation of lactate (maximum inhibition 10 p.c.), the former always being rather more effective than the latter (Table 8). Although dilutions down to 10^{-5} M were examined, no stimulatory effects could be observed with either inhibitor. Evidence as to the exact site of action of these substances (i.e. glycolysis or oxidation) was not as clear-cut as with some of those described above.

TABLE 8.

The effect of nitrophenols.

As for Table 7.

Inhibitor	Concentration (M)	Conditions*	Overall percentage inhibition
2:4-dinitro-phenol	5×10^{-3}	1	61
2:4-dinitro-phenol	5×10^{-3}	2	58
2:4-dinitro-phenol	5×10^{-3}	3	68
p-nitro-phenol	5×10^{-3}	1	29
p-nitro-phenol	5×10^{-3}	2	36
p-nitro-phenol	5×10^{-3}	3	45
p-nitro-phenol	10^{-3}	3	11
2:4-dinitro-phenol	10^{-3}	3	27

* See Table 2.

8-Hydroxy-quinoline, p-phenylene-diamine, dipicrylamine and picrolonic acid.

These substances are known selectively to remove Mg++, Fe+++, K+ and Ca^{++}, respectively. They were, therefore, tested in the M.B. respiratory system to see whether a metal-dependence could be assumed. In the case of dipicrylamine, the experiments were unsuccessful because of the interaction of this substance with M.B., apparently causing irreversible removal of the dye and therefore 100 p.c. inhibition of respiration.

The other three all inhibited O_2 uptake with glucose as substrate very strongly (Table 9), but that of lactate only weakly or not at all. These findings show that Ca++ and Mg++ are necessary,[3] certainly for the preliminary transformation of glucose, and possibly also for its oxidation.

TABLE 9.

The effect of metal-precipitants.

As for Table 7.

Inhibitor	Concentration (M)	Substrate	Conditions*	Overall percentage inhibition
8-hydroxy-quinoline	5×10^{-3}	Glucose	3	70
8-hydroxy-quinoline	10^{-3}	Glucose	3	35
8-hydroxy-quinoline	2×10^{-3}	Glucose	2	37
8-hydroxy-quinoline	2×10^{-3}	Glucose	3	46
8-hydroxy-quinoline	5×10^{-3}	Lactate	1	22
Picrolonic acid	$1{\cdot}25 \times 10^{-3}$–	Glucose	3	63–48
Picrolonic acid	$2{\cdot}25 \times 10^{-4}$			
Picrolonic acid	$2{\cdot}25 \times 10^{-4}$	Lactate	3	24–23
Picrolonic acid	$6{\cdot}2 \times 10^{-5}$	Glucose	3	39
Picrolonic acid	$6{\cdot}2 \times 10^{-5}$	Lactate	3	1
p-phenylene-diamine	$3{\cdot}3 \times 10^{-3}$	Glucose	3	45–55
p-phenylene-diamine	$3{\cdot}3 \times 10^{-3}$	Lactate	3	0–10

* See Table 2.

Other Inhibitors.

Hydroxylamine and azide were tested. The former behaved like M.B., i.e. caused haemiglobin formation, and thereby set up an O_2 uptake. The latter, in the presence of M.B. and cells, caused evolution of a gas (apparently N_2) due to its oxidative decomposition. It was not possible to determine the effect of this substance quantitatively, but qualitative inhibition of glucose oxidation, and apparently no effect on lactate, seemed indicated.

Malonate was inactive, showing non-participation of succinoxidase; cyanide, pyrophosphate, thioglycollate, sulpha drugs, penicillin and benzoate all failed to give significant inhibitions on either glucose or lactate.

DISCUSSION.

The results of this investigation provide further evidence for the intimate connection which exists between phosphorylations and oxidations in the erythrocyte system supplemented with methylene blue.

[3] Since the completion of this work, Stickland (1949) has confirmed the necessity of Mg++ and another metal for phosphoglucomutase activity.

Hexokinase inhibitors (quinine, caffeine and phloridzin) and substances interfering in glycolysis (fluoride and iodoacetate) all severely affect the O_2 uptake with glucose as substrate, once again proving the necessity for an initial "activation" of the hexose prior to oxidation. The fact that most of the inhibitors found effective against glucose were inactive towards lactate oxidation would suggest that it is this preliminary activation, and not the oxidative enzymes, which is sensitive.

The necessity for Mg^{++} and Ca^{++} in glucose metabolism is also not surprising; the possibility that the metal-removing inhibitors act by non-specific attack on various enzymes, however, cannot be excluded.

The results explain certain earlier findings more clearly: Preincubation of glucose with the cells prior to M.B. addition (Method II, Nossal, 1948) leads to an accumulation of glycolytic intermediaries from the hexose phosphates to lactic acid, thus giving rise to a comparatively larger O_2 uptake when M.B. is added; the hexokinase reaction seems the limiting factor, and also that most sensitive to various inhibitors.

This work also shows that respiration with glucose as substrate can definitely not be ascribed *in toto* to initial conversion to lactate, followed by oxidation of the latter to pyruvate, otherwise addition of the latter would have a more pronounced inhibitory effect; apparently the main O_2 consuming reaction is an oxidative degradation of a hexose phosphate.

The respiration studied here shows several peculiarities, e.g. insensitivity to cyanide and malonate, both of which affect a wide range of animal tissues; nicotine insensitivity of the lactate oxidation, whereas glucose oxidation is affected (the reverse from other tissues); the resistance of the oxidative enzymes (e.g. haemiglobin reductase) to most of the inhibitors, as judged from the oxidation of lactate (it seemed remotely possible that lactate oxidation might be independent of the reductase; however, since Kiese has shown reduction of haemiglobin running parallel with lactate oxidation, this explanation must be ruled out).

The nature of this reductase remains a mystery: Kiese (1944) and Gibson (1948) suggest that it is a flavoprotein; since no general flavoprotein inhibitors are known, it is not possible to test the claim from that angle; separation of the enzyme does not seem to have been attempted. The products of hexose phosphate oxidation have also been largely a matter of surmise.

It is possible that some of the inhibitors of glucose oxidation act by altering either cell membrane permeability or the absorption rate of glucose into the cell; the first suggestion seems ruled out because of the fact that lactate oxidation is generally unaffected, but the second cannot be denied as a possibility.

An extension of this work to haemolyzed erythrocytes will be reported shortly, which augments and further clarifies findings recorded here.

SUMMARY.

A series of inhibitors has been tested in the oxidations of glucose and lactate by rabbit erythrocytes and methylene blue. The results strongly support the earlier contention that glucose must be phosphorylated prior to direct oxidation.

The following have been found active against glucose, the mechanism of action being suggested in parentheses:

Quinine, caffeine and phloridzin (hexokinase).

Iodoacetate and fluoride (two essential steps in glycolysis, which must occur for glucose phosphorylation to proceed).

8-hydroxyquinoline, picrolonic acid and p-phenylene diamine (removal of essential metals, such as Mg^{++}, Ca^{++} and possibly Fe^{+++}).

2:4-dinitrophenol and p-nitrophenol, azide, nicotine, sodium di-ethyl-dithiocarbamate are all active, but the sites of action are less definite.

Cu^{++} was the only inhibitor which seemed to affect the oxidative system itself.

Pyruvate was the only inhibitor which markedly retarded lactate oxidation; it was also effective against glucose, but much less strongly.

The following were tested and found inactive in both systems: Cyanide malonate, pyrophosphate, hydroxylamine, thioglycollate, sulpha drugs, penicillin and benzoate.

The bearing of these results on the mechanism of methylene blue respiration is discussed.

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THE METABOLISM OF ERYTHROCYTES

VI. ON THE RESPIRATION OF CELL HAEMOLYSATES[1]

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It is well-known that haemolysis destroys the ability of non-nucleated erythrocytes to respire on glucose in the presence of methylene blue (M.B.), (Barron and Harrop, 1928). Since a preliminary phosphorylation is necessary before glucose can serve as a substrate for oxidation (Barron, 1929; Warburg and Christian, 1931 a, b; Moruzzi, 1936, 1937 a; Nossal, 1948, 1949), the most obvious explanation of this finding is destruction of the hexokinase system. This is supported by the findings that in the presence of M.B. (a) lactate can still be oxidized by lysed cells (Kiese, 1944) and (b) phosphorylated hexoses are readily oxidized (Warburg and Christian, 1931 a, b; Kiese, 1944; Gibson, 1948).

Following the investigation of a series of inhibitors for the oxidation of glucose and lactate by intact cells (Nossal, 1949) the respiration of haemolysates has been subjected to the same tests, and the effectiveness of different hexose phosphates compared.

Methods.

The only procedure not described in previous communications is the preparation of haemolysates. Fresh defibrinated rabbit blood was spun down, the cells washed with phosphate saline, spun down again, and then lysed with glass-distilled water containing certain amounts of nicotinic acid amide (see below). The volume of water used for lysis was roughly twice the cell volume, and the exact concentration of each sample was recorded as haematocrit (which was maintained between 30 and 35 for most experiments).

Oxygen uptakes were measured in standard Warburg equipment at 38° C., and the pH of all solutions adjusted with a glass electrode pH meter.

RESULTS.

I. General Observation.

Optimum Conditions of Lysis.

When distilled water alone is used for lysis, it can be shown that haemolysates thus prepared rapidly lose the ability to oxidise either lactate or hexose phosphates in the presence of M.B.

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This has been ascribed to the enzymatic hydrolysis of pyridine nucleotides, which are the essential hydrogen acceptors of "Zwischen-ferment" (Warburg and Christian, 1931 b) and lactic dehydrogenase. Such destruction may be off-set by either adding Coenzyme I or specifically inhibiting the responsible phosphatase with nicotinamide. Gutmann *et al.* (1947) used a final concentration of 0·16 M (i.e. 160 mM) nicotinamide during lysis in their experiments.

After repetition of their experiments, it seemed as though the amount of nicotinamide used was much too large, and the effect of its concentration during lysis on the subsequent respiratory activity of haemolysates was fully investigated. As can be seen from Table 1, there is a pronounced *decrease* in the ability of samples to oxidise hexose monophosphate when a certain concentration of nicotinamide was exceeded. Hence, lysis was performed usually with nicotinamide solutions which would give a final concentration of $1–2 \times 10^{-2}$ M.

TABLE 1.

The effect of nicotinamide concentration during lysis on the subsequent oxidation of hexose phosphate.

Substrate: $2·5 \times 10^{-3}$ M (final). Each cup contains 2·0 ml. haemolysate, 0·1 ml. N NaOH (centre well), 0·2 ml. M.B., 0·2 ml. substrate (hexose monophosphate*) and 0·5 ml. phosphate-saline pH 7·4. The values represent total oxygen uptake (μl.) for the experimental period of 1½ hours. The various haemolysates in each experiment are of the same concentration and were prepared from the same blood sample.

Exp. No.	Final nicotinamide concentration during lysis (M)					
	0·2	0·1	0·05	0·02	0·01	0
1	79	110	145	167	186	45
2	38	96	136	152	156	16
3	70	92	—	133	154	89
4	113	175	204	—	—	35
5	—	152	214	224	197	— —
6	—	216	254	263	250	— —

* Hexose monophosphate is the Ba salt of a mixture of two parts glucose–1–phosphate and one part glucose–6–phosphate.

TABLE 2.

The effect of nicotinamide during lysis on lactate oxidation.

Substrate: 3×10^{-2} M. Otherwise as Table 1.

System	Concentration of nicotinamide during lysis (M)	Total O_2 uptake (μl.)
Haemolysate	0·2	56
Haemolysate	0·15	59
Haemolysate	0·1	51
Haemolysate	0·05	32
Haemolysate	0·025	15
Cells	—	202

In the case of lactate oxidation, however, a different set of results is obtained. In this case, higher concentrations of nicotinamide favour more extensive lactate oxidation. This discrepancy is difficult to explain if intervention of Coenzymes I or II in both the oxidations is stipulated.

Under the conditions employed generally for lysis, lactate oxidation is almost negligible. Even increases of nicotinamide concentration up to 0·2 M raise oxygen uptakes of haemolysates to values which are only fractions of those observed with intact cells of the same concentration from the same blood sample (Table 2).

Concentrations of lactate from 6×10^{-2} M to 3×10^{-3} M were examined with haemolysates active in oxidizing hexose monophosphates, and the oxygen uptakes were all almost negligible. It may therefore be assumed that, under these conditions, no appreciable part of the oxygen uptake is due to lactate oxidation.

The Oxidation of Various Hexose Phosphates.

The following samples were examined: glucose-1-phosphate (G–1–P), hexose-phosphate (H–M–P), fructose-6-phosphate (F–6–P) and fructose-1:6-diphosphate (H–D–P). Concentrations used ranged from $5\text{–}1 \cdot 25 \times 10^{-3}$ M.

The first and most noticeable difference between the four is the much lower O_2 uptake observed with the diphosphate, when compared with the three monophosphates. The ratio of O_2 uptake is usually more than 3:1 in favour of the monophosphates (Table 3). Whether this can be ascribed merely to lower solubility of the diphosphate it is difficult to say, but it does not seem likely. Rather, it would appear as though one or more of the monophosphates could be oxidized as such, whereas, when glycolysis had proceeded as far as the diphosphate, no oxidation could occur until further conversion to the less oxidizable triose phosphate or lactate had taken place.

TABLE 3.

The oxidizability of various hexoses phosphates.

Conditions: as in Table 1.

Substrate	Total O_2 uptake (μM)
Glucose–1–PO_4 (G–1–P)	8·7
"Hexose–PO_4" (H–M–P)	9·7
Fructose–6–PO_4 (F–6–P)	10·1
Fructose–1:6–diPO_4 (F–1–D–P)	2·7

The Effect of Preincubation on the Oxidizability of Hexose Phosphates.

In the oxidation of glucose by intact erythrocytes + M.B., increases in O_2 uptake were observed when substrate and cells were incubated together prior to addition of M.B. (Nossal, 1948). This was ascribed to the formation of phosphorylated intermediaries and lactic acid, which constitute the substrates for the various oxidations inhibited by addition of M.B.

The four hexose phosphates, being intermediaries in the glycolytic path, might well be expected to be converted eventually to lactic acid, and its other 3–C precursors. It was therefore decided to determine whether increases or decreases in O_2 uptake result from preincubation. Experiments were set up in which for each hexose phosphate, the O_2 consumptions with (Method II, Table 4) and without (Method I, Table 4) preincubation were compared.

Although quantitatively the increases or decreases observed for a given substrate differed widely, the qualitative differences were very pronounced, except in the case of fructose-1:6-diphosphate, in which O_2 uptakes were very small by either method.

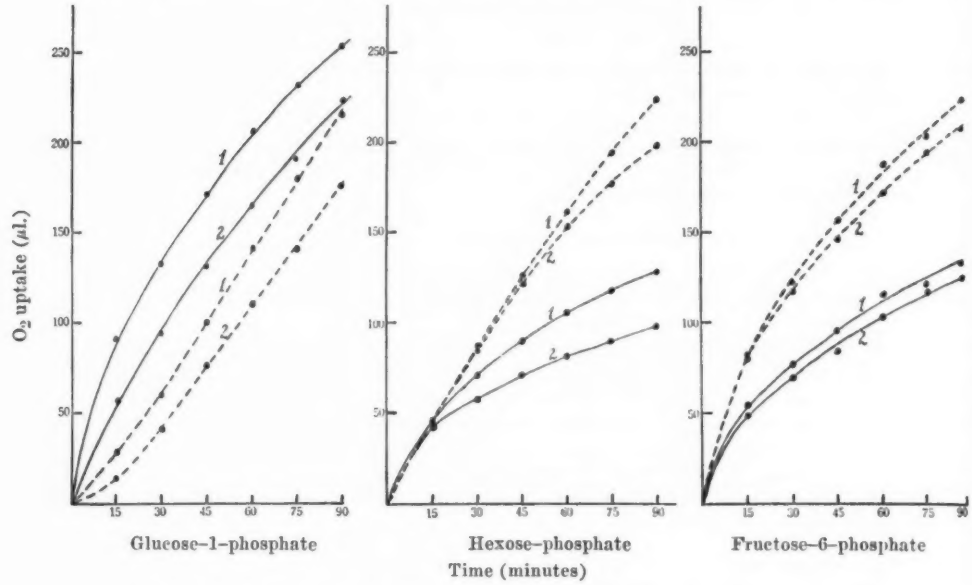


Fig. 1. The effect of preincubation on the oxidation rates of hexose monophosphates. Each part shows the results of two distinct experiments, 1 and 2. The dotted curves represent O_2 uptakes for cups where the substrate had been preincubated with the cells for 1 hour, the unbroken lines O_2 uptakes for cups in which the substrate had been pipetted into a side-bulb. M.B. was added to both cups simultaneously from the other side-bulb.

TABLE 4.

The effect of preincubation on the oxidizability of hexose phosphates.

Conditions: Substrate and M.B. as in Table 1; substrates in one side-bulb, M.B. in the other. For Method II, substrate tipped in after equilibration, for Method I not till 1 hour later. In both, M.B. tipped in 1 hour after the first tip. Experimental period (after M.B. tip): 1½ hours.

Substrate	Method	Total O_2 uptake (μM.)
G–1–P	I	8·0
G–1–P	II	11·1
H–M–P	I	8·4
H–M–P	II	5·4
F–6–P	I	9·2
F–6–P	II	5·6
H–D–P	I	1·7
H–D–P	II	2·3

From some typical results cited in Table 4, it would appear as though G–1–P and H–D–P are converted to other substances prior to oxidation (since there is an increase in uptake for Method II) whereas F–6–P and the G–6–P (in "hexose-phosphate") are oxidized as such.

The quantitative differences in the ratio O_2 uptake (Method I), O_2 uptake (Method II) for several experiments can then be explained by variations in the glycolytic activity of the individual haemolysate samples.

When the *rates* of O_2 consumption are examined, it can be seen that with "H–M–P" the *initial* uptake for Method II sometimes exceeds that for Method I, although over-all uptakes for the latter are greater than for the former (Fig. 1). This is opposed to the behaviour of F–6–P, in which Method I shows much higher uptakes continuously. In the case of G–1–P, initial uptakes by Method II are again much higher, the rate then diminishing, whereas, for Method I, initial uptakes are very low, the rates then increasing, but never quite catching up on Method II.

The Comparative Oxidizability of Hexose Monophosphates.

Using approximately equal concentrations of all substrates, the O_2 uptakes of the three monophosphates were compared by both Methods I and II.

From Table 5 it can be seen that for both methods, the uptakes are lowest for H–M–P. For Method I, G–1–P and F–6–P are almost equal, the latter exceeding the former more frequently, but for Method II, uptakes on G–1–P are always much greater than on F–6–P.

TABLE 5.

Comparison of the oxygen uptakes on the three hexose monophosphates.

Conditions: As in previous tables.

Experiment No.	Method	Total O_2 uptakes (μM.)		
		Glucose–1–PO_4	"Hexose-PO_4"	Fructose–6–PO_4
1	I	9·5	8·0	9·3
2	I	—	7·8	8·8
3	I	7·8	—	10·0
4	I	8·3	—	7·5
5	I	8·0	—	9·7
6	I	10·0	—	11·1
7	II	11·3	4·25	5·5
8	II	12·5	5·35	7·3
9	II	7·3	2·7	3·2
10	II	10·0	—	5·9
11	II	11·0	—	8·7
12	II	14·1	—	9·7
13	II	8·2	—	5·5

That H–M–P should be less oxidizable than the other two is rather unexpected. This finding could be accounted for by assuming either that the sample was less pure (and therefore the concentration lower) or that some decomposition occurred during the dissolving of the mixed Ba++ salt (the other two phosphates were: K–glucose–1–PO_4 and free fructose–6–PO_4, and therefore required no treatment for solution).

The approximate equality of O_2 uptakes on G–1–P and F–6–P is compatible with the suggestion that the former is oxidized as glucose–6–phosphate by Zwischen ferment (Warburg and Christian, 1931 b) and the latter as such by possibly another, similar oxidative enzyme.

This would then also explain the increased effectiveness of G–1–P on preincubation (partial conversion to G–6–P, and F–6–P) and the decreased uptakes of F–6–P on incubation (because of conversion to the non-oxidizable H–D–P, and the less oxidizable triose-phosphate and lactic acid).

The small uptakes on H–D–P are probably due to the fact that it must first be converted to triose-phosphate and also to lactic acid, before any oxidations occur. In the case of the latter, especially, uptakes on concentrations below 10^{-2} M are very small.

II. The Effect of Inhibitors.

Substrates used throughout this section were H-D-P and H-M-P, unless otherwise stated. Only inhibitors found effective against glucose or lactate oxidation in intact erythrocytes (Nossal, 1949) were examined. The effects of each on glucose and lactate oxidation are indicated in parentheses.

Phoridzin, quinine and caffeine: (These were all strongly inhibitory with glucose as substrate, and ineffective with lactate). Quinine showed no effect with either hexose ester. This suggests that its previous action can be entirely accounted for by inhibition of glucose phosphorylation.

Phloridzin showed a rather weak inhibition against both phosphates, only once exceeding 10 p.c. in many experiments. Caffeine inhibited H–M–P oxidation 5–15 p.c., and that of H–D–P, 20–30 p.c.

Therefore, the earlier claim, that the first two substances affect primarily the hexokinase system, has been further substantiated. In the case of caffeine, one or more of the steps between H–D–P and lactate may also be sensitive.

Iodoacetate and fluoride: (both severely reduced O_2 uptake with glucose as substrate, but did not affect that with lactate).

Iodoacetate had a peculiar effect on H–M–P oxidation: When substrate, M.B. and inhibitor were added simultaneously, virtually no reduction in O_2 uptake (compared with uninhibited controls) was observed over the experimental period of 1½ hours. Occasionally, inhibitions up to 10 p.c. were recorded, but more often no effect was noticeable. However, when the haemolysates were preincubated with iodoacetate for an hour, and *then* substrate and M.B. were added, inhibitions up to 96 p.c. for $3–5 \times 10^{-3}$ M iodoacetate were found.

The extent of inhibition can be shown to depend on the contact period of the inhibitor with the haemolysate: In a typical sample, an inhibition of 80 p.c. was observed when the preincubation period was one hour, whereas with 15 minutes' preincubation it was only 19 p.c.

The absence of inhibition without preincubation agrees with Kiese's results (1944) who observed that the reduction of haemiglobin by hexose monophosphate was insensitive to iodoacetate.

From the above results and from the fact that iodoacetate is known to inhibit triose phosphate dehydrogenase, the effect of the inhibitor in erythrocytes can be considered as of a dual nature: It not only inhibits triose phosphate dehydrogenase (thereby affecting indirectly the phosphorylation of glucose), but after prolonged contact also strongly inhibits the hexose monophosphate oxidations (similar effects to those described above have been found with F–6–P as substrate).

Fluoride exerted virtually no inhibition on H–M–P oxidations. 10^{-3} M iodoacetate inhibited respiration on H–D–P 100 p.c., and 10^{-5} M up to 57 p.c., showing that glycolytic conversion to C_3 compounds must occur prior to oxidation. Fluoride also inhibited strongly, but

never more than 60 p.c. The combined effects shown by these inhibitors suggest that H–D–P can be oxidized only after initial conversion to 3-carbon compounds.

Copper: (the only inhibitor suggested to affect the oxidative system itself in the earlier work) had a pronounced effect on H–M–P oxidation. Inhibition up to 68 p.c. for 10^{-3} M Cu++ was observed.

In the case of H–D–P 100 p.c. inhibition occurred in all cases. Actually, an output of gas was observed in all experiments with 10^{-3} M Cu++, resembling that found with glucose oxidation by intact cells. This gas is possibly oxygen, liberated from oxyhaemoglobin during its conversion to haemiglobin.

Therefore, it appears as though Cu++ does attack the oxidative enzymes (Zwischen ferment or haemiglobin reductase), and that both triose-phosphate dehydrogenase or the enzymes *preceding* lactic dehydrogenase (the latter is not so obviously affected, since oxidation of lactate is only slightly inhibited) are also sensitive.

The inhibition drops very sharply for 10^{-4} M Cu++; so sharply, in fact, that one might still be tempted to ascribe the effect of the metal to partial oxidation of the substrate. However, the *total* inhibition of respiration on H–D–P points to some other explanation.

The nitrophenols: (both p–nitro– and 2:4–dinitro–phenol were active in inhibiting glucose oxidation). 2:4–dinitro–phenol was found considerably more active against both H–M–P and H–D–P. At 5×10^{-3} M, inhibitions from 45–66 p.c. were observed, at $2{\cdot}4 \times 10^{-3}$ M the inhibition was halved, but that due to p–nitro–phenol was now negligible.

This suggests that 2:4–dinitro–phenol, at least, has a direct effect on the oxidative processes, whatever else its action on the glycolytic reaction might be.

Diethyldithiocarbamate: (which inhibited glucose, but not lactate oxidation in intact erythrocytes) had no effect on H–M–P oxidation but at a concentration of 2×10^{-3} M inhibited O_2 uptake on H–D–P *ca* 20 p.c.

8–hydroxy–quinoline, p–phenylene–diamine and picrolonic acid: (all strongly active against glucose, and inactive against lactate).

The first caused only weak inhibition (maximum 15 p.c.) of O_2 uptake on either substrate; the inhibition by p–phenylene diamine was of the same order for H–M–P but much more pronounced for H–D–P.

$2{\cdot}5 \times 10^{-4}$ M picrolonic acid on H–M–P showed a very strong inhibition, ranging from 59–78 p.c. But on H–D–P, only slight effects were observed (up to 15 p.c.). This is the only inhibitor towards which H–M–P oxidation is more sensitive than that of H–D–P. It suggests that the systems responsible for oxidation of G–6–P and possibly F–6–P both require Ca++, unless the substance acts in some other manner.

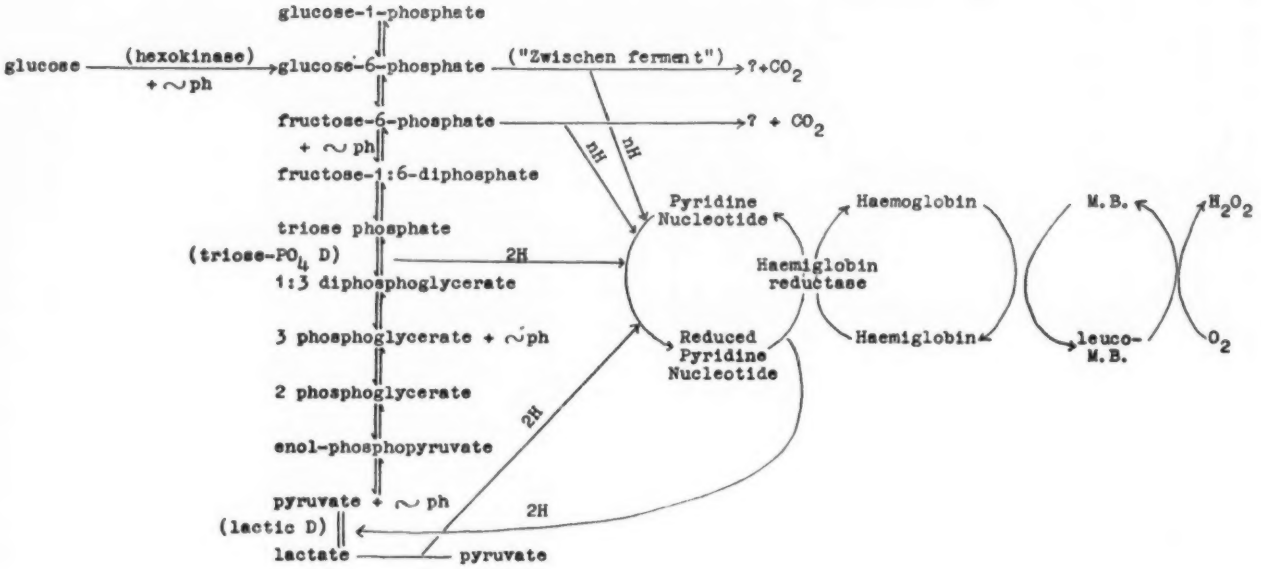
Azide: As noted in the previous communication, the examination of this inhibitor is complicated by the fact that it itself seems to be oxidatively decomposed. Gas outputs up to 80 μl. have been observed for 5×10^{-3} M azide. This would represent a balance of unknown O_2 uptakes and N_2 outputs. Correcting for blanks, it appears as though azide has no effect on H–M–P oxidation, but a pronounced inhibition is observed with H–D–P. The results, however, are at the best qualitative.

Nicotine: (which definitely inhibited glucose oxidation but not that of lactate) had little effect with either H–M–P or H–D–P as substrate. This shows that its action in the erythrocyte is primarily on the glucose phosphorylating system.

Pyruvate: (inhibited lactate oxidation severely and that of glucose markedly). The lowering of oxygen uptake on H–M–P depends very definitely on experimental conditions. When inhibitor, substrate and M.B. are added to the cells simultaneously, no inhibition whatever is observed. However, when H–M–P and pyruvate are preincubated with the cells for an hour prior to M.B. addition, an appreciable reduction in O_2 uptake is found.

SCHEME I

The connection between glycolysis and M.B. respiration.



The initial reason for examining the effect of pyruvate may be stated as follows: It was suspected that, during the preincubation, some H–M–P would be converted to lactate. The oxidation to pyruvate of the comparatively low concentrations of lactate thus formed would be almost entirely inhibited by the added pyruvate. Therefore, the observed pyruvate inhibition would be a measure of the extent of conversion of H–M–P to lactate.

Actually, the finding that lactate is virtually not oxidized by haemolysates under the existing experimental conditions excludes this interpretation of the pyruvate effect.

It is also known that pyruvate acts as a hydrogen acceptor to the triose phosphate dehydrogenase system. Hence, addition of pyruvate should accelerate oxidation of triose phosphate, and therefore, indirectly, decomposition of hexose phosphates. Hence, the lowering of O_2 uptake observed when G–1–P, H–M–P, and F–6–P are preincubated with pyruvate prior to M.B. addition is explained not by inhibition of the oxidation of lactate formed glycolytically, but rather by the fact that far more hexose phosphate has been decomposed anaerobically; i.e. the O_2 consumption after preincubation is a measure *not* of the oxidation of C_3 compounds formed glycolytically, but of residual hexose monophosphate.

The extensive reduction of O_2 uptake on H–D–P caused by pyruvate could be explained along similar lines: Since lactate seems non-oxidizable by the system, O_2 uptake after M.B. addition may be due to aerobic triose phosphate oxidation. In the presence of pyruvate, some of the oxidation will proceed anaerobically, pyruvate being the hydrogen acceptor, so that O_2 uptake will be decreased.

The action of pyruvate may therefore be summarized as follows: It has no effect on the direct oxidation of any of the hexose monophosphates (and thus it seems unlikely that pyruvate is an end-product of this oxidation). It decreases O_2 uptake when preincubated with hexose monophosphates by accelerating their anaerobic removal. It decreases O_2 uptake on H–D–P by diverting to a large extent the acceptance of hydrogen (arising from the dehydrogenation of triose phosphate) from O_2 to pyruvate.

DISCUSSION.

In non-nucleated erythrocytes, all the steps of Meyerhof's phosphorylative glycolysis occur, except that the starting product is glucose rather than glycogen.

The intimate connection between this process and M.B. respiration, or the reduction of haemiglobin, has been amply demonstrated (Scheme I). The main problems have been the exact nature of substrates which could be oxidized, and of their end-products.

The following have been shown to increase oxygen uptake in the presence of M.B., or to accelerate the reduction of haemiglobin: Glucose, other hexoses, fumarate and malate (Kiese. 1944; Nossal, 1948), pentoses (Nossal, 1948) and lactate, all in intact cells; glucose-6-phosphate (Warburg and Christian, 1931 b), triose phosphate, glyceraldehyde (Moruzzi, 1937 b) and lactate (Wendel, 1933) in haemolysates.

In this work, it is shown that fructose-6-phosphate can also be considered as a primary substrate, whereas glucose-1-phosphate would appear to be oxidized after initial conversion to one or both the 6-phosphates.

M.B. respiration is a complex process, because portion of any hexose phosphate studied as a primary substrate will inevitably be converted to all the glycolytic intermediaries between itself and lactate. Thus, the subsequent oxygen uptake will be the sum of a number of individual processes.

Although it is difficult to assess the relative importance of direct hexose phosphate and of C_3 compound oxidation in intact cells, the problem is somewhat simpler in haemolysates: Here the bulk of the total oxygen consumption is due to direct hexose phosphate oxidation; this is shown (a) by the finding that hexose diphosphate causes such low O_2 uptakes and (b) by the lowering of O_2 uptake observed when H-M-P and F-6-P are incubated with the haemolysate prior to M.B. addition: Conversion of C_6 to C_3 compounds is known to occur, and lactate is not oxidized appreciably under the conditions employed (it was not possible to examine triose phosphate oxidation); therefore, the decreases give a measure of anaerobic hexose monophosphate removal, and the observed O_2 uptake is probably due to residual hexose monophosphate.

A certain amount of knowledge is also accumulating about the end-products of the various oxidations: Lactate is converted to pyruvate, which is not further utilized (Wendel, 1933); glyceraldehyde to glycerate (Moruzzi, 1937 b) and triose phosphate to phosphoglycerate. Warburg *et al.* (1930) claimed that pyruvate was one of the end-products of glucose oxidation; this seems unlikely since excess pyruvate addition has no effect on the direct oxidation of hexose monophosphate; it is more probable that the pyruvate which appeared was due to the oxidation of glycolytically formed lactate. Dickens (1938) described the first steps of a direct, phosphorylative oxidation of glucose in erythrocytes, and his mechanism has also been suggested by Gibson (1948). Results of work in this laboratory (unpublished) suggest that several molecules of oxygen are taken up, and CO_2 produced, for each molecule of hexose utilized. Since there is no evidence of the existence of the tricarboxylic acid cycle, it is quite possible that a mechanism for the "phosphogluconic acid shunt" is called into operation by M.B. addition (or the presence of haemiglobin).

The results of inhibition experiments have to some extent been discussed throughout the text and in the previous communication (Nossal, 1949). Table 6 gives a summary of results for each inhibitor tested against four different systems. It can be seen that lactate oxidation is peculiarly insensitive, the only active inhibitor being pyruvate. Glucose utilization is by far the most sensitive, this no doubt being due to the *indirect* effect of many inhibitors (e.g. iodoacetate and fluoride) or hexokinase. H-M-P oxidation as such is affected mainly by Cu^{++}, picrolonic acid and iodoacetate (if the haemolysate is first incubated with the latter). Oxygen consumption on H-D-P is more sensitive because again preliminary anaerobic conversions must occur before any oxidations can take place, so that many sites of action are open to inhibitors.

TABLE 6.

Summary of the effects of various inhibitors on M.B. respiration.

The number of + signs gives an approximate comparison of the extent of inhibition.

System Substrate	Intact cells Glucose	Intact cells Lactate	Haemolysate H-M-P	Haemolysate H-D-P	Suggested site of action
Azide	++	0	0	++	unknown
Caffeine	+++	0	+	++	glucose phosphorylation; may have other effects.
Copper	++++	+	++	++++	cell membrane; direct effect also on oxidative enzymes.
Diethyldithiocarbamate	++	0	0	++	unknown; may be aldolase.
Fluoride	+++	0	0	++	only on glycolytic processes (enolase).
8-hydroxy-quinoline	+++	0	+	+	on an anaerobic step requiring Mg++.
Iodoacetate	++++	0	0—+++(1)	++++	directly on both glycolytic and oxidative enzymes; indirectly on hexokinase.
Nicotine	++	0—+	0	+	mainly on glucose phosphorylation.
p-nitrophenol	++	0	0—+	0—+	apparently on phosphorylation.
2:4-dinitrophenol	+++	+	+++	++	as above, but may have multiple effect.
p-phenylene diamine	+++	0	+	+	apparently mainly on phosphorylation.
phloridzin	+++	0	0	+	mainly on hexokinase.
Pierolonic acid	+++	+	+++	+	apparently on H-M-P oxidative enzymes.
Pyruvate	++	++++	0—+++(2)	++	on lactic dehydrogenase, or by causing more rapid removal of H-M-P.
Quinine	+++	0	0	0	only on hexokinase.

(1) Inhibition is observed only when the inhibitor is preincubated with the haemolysate prior to substrate and M.B. addition.

(2) Inhibits only when preincubated with haemolysate and substrate prior to M.B. addition.

SUMMARY.

Investigation of the effect of nicotinamide concentration during the lysis of rabbit erythrocytes showed that the methylene blue (M.B.) respiration of the haemolysates was optimal for low ($1–2 \times 10^{-2}$M) concentrations of nicotinamide when hexose monophosphate (H-M-P) was used as a substrate; higher concentrations, however, favour lactate oxidation. Under routine lysis conditions, lactate oxidation is almost negligible.

The M.B. respiration of haemolyzed erythrocytes with various hexose monophosphates as substrates was several times higher than when fructose-1:6-diphosphate (H-D-P) was used.

Results of preincubation studies (in which haemolysates + substrates were incubated for an hour prior to M.B. addition) suggest that both glucose-6-phosphate and fructose-6-phosphate are oxidized as such, that glucose-1-phosphate may first have to be converted to either of these, and that H-D-P is oxidized only after splitting to 3-carbon compounds.

Iodoacetate causes a gradual but finally almost complete inhibition of H-M-P oxidation. This is observed only after incubating enzyme and inhibitor for some time. It also completely nullifies oxygen uptake on H-D-P. Fluoride has no effect on H-M-P oxidation, but strongly affects that of H-D-P.

Phloridzin and quinine did not markedly inhibit H-M-P or H-D-P oxidations, but caffeine had a definite action on both.

Copper in fairly high concentrations caused a strong reduction of oxygen uptake on H-M-P, and a 100 per cent. reduction on H-D-P.

Both m-nitrophenol and 2:4 di-nitro-phenol inhibited both oxidations, the latter considerably more strongly than the former.

Diethyldithiocarbamate, 8-hydroxy-quinoline and p-phenylene diamine all had only weak effects on both processes, except that the last-named more strongly inhibited H-D-P oxidation. Picrolonic acid very markedly inhibited H-M-P oxidation. Azide did not seem to affect H-M-P, but inhibited respiration on H-D-P. Nicotine had little effect on either process.

Pyruvate decreased oxygen uptake on hexose monophosphates by increasing their anaerobic removal, and on H-D-P by diverting hydrogen acceptance from oxygen. Pyruvate had no direct effect on H-M-P oxidation, and is therefore probably not an end-product.

The mechanisms of M.B. respiration in erythrocytes are discussed in the light of the above findings.

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STUDIES ON MUSCLE WITH THE ELECTRON MICROSCOPE

1. THE ULTRASTRUCTURE OF TOAD STRIATED MUSCLE

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A muscle fibre is generally believed to develop from the fusing together of many cells. The cytoplasm of this giant cell is then organized in such a way that a general property of all protoplasm, namely contractility, is accentuated. It should not be forgotten that the muscle cell also exhibits the other general properties of cytoplasm. Consequently, in any interpretation of the ultrastructure of muscle it does not follow that all features are necessarily related to contraction.

Monné (1948) and other cytologists support the thesis that cytoplasm is essentially organized on a fibrous basis and that it is in such a matrix that the living process functions. The chromosome is an example of a cellular constituent with an obvious longitudinal organization. Under certain conditions cytoplasmic fibrils can be formed into cross-striated bundles which resemble strikingly the appearance of striated muscle (Monné, 1948). Thus the muscle fibre can be regarded as the fusion of many cells, the fibrous protein systems having been organized into long parallel sheets and bundles instead of the random or radial arrangement found in single cells.

Classical histology provides a clear picture of the striated muscle fibre structure as seen externally. A long sarcolemmal sheath, 20–100μ in diameter and from 1·5–30 cm. long, contains material which displays regular striations. The subdivision into various components was successfully investigated in insect muscles where the cross striations are on a larger scale. In the living fibre these striations could be seen to run around the fibre, yet in cross section or in teased preparations the muscle protoplasm could be split into smaller components, each displaying the characteristic striations. In transverse sections of some muscles roughly hexagonal cleavage planes called Cohnheim areas were described. Between these areas sarcoplasm was supposed to exist. Some

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investigators postulated further that on the periphery of the muscle fibre were to be found rapidly contracting fibrils or tetanus fibrils while those in the interior were slowly contracting tonus fibrils (Bozler, 1928). Others contended that the sarcoplasm was capable of contraction as well as, or instead of, the fibrils. The rôle of the sarcolemmal sheath in contraction has been studied by Ramsay and Street (1947) but the structural nature of the sheath is still obscure. Barer (1947) has recently obtained interesting results using a micromanipulator.

As will be seen, the knowledge concerning the more intimate structural details of striated muscle is becoming considerable, and the link between the expenditure of chemical energy and the performance of mechanical work is becoming clearer as the result of recent biochemical investigation into the relationship between the high-energy phosphate bonds existing in adenosine triphosphate and the actin-myosin protein systems. Thus, the muscle fibre may well be the first biological tissue to yield fundamental knowledge concerning the rôle of protein systems in the life process.

Materials and Methods.

In any given sample of material mounted on a grid and ready for examination with the electron microscope (E.M.) there is represented a very small fraction of the whole. The method of preparation used in this investigation can only be described as drastic. Even after the gross preparation the subsequent drying in air and desiccation *in vacuo* must impose conditions which are not constant from sample to sample. The effect of the beam of electrons on the material to be examined depends on many factors, such as intensity, rate of increase of intensity, thickness of the material, staining technique, all of which are not easily controlled from sample to sample. These and other factors necessitate the examination of many samples before any definite idea can be obtained of the overall structure. Despite the examination of hundreds of preparations, the way in which significant small additions to the details of the overall picture still keep presenting themselves as examination proceeds, emphasizes the minute fraction of material which is examined, and also encourages investigation to proceed, notwithstanding the apparently complete picture which has appeared to date.

In view of the above considerations, and because by experience the muscle tissue of the toad, *Bufo marinus*, could be reduced by a standard procedure to a form suitable for electron microscopical examination, all the data to be presented are from this animal. This has some disadvantages, the chief of which is that any generalization made concerning muscle tissue may not be valid. However, there is strong presumptive evidence that extrapolation to other striated muscles will be valid at the macromolecular level of organization. The main details of the ultrastructure to be discussed have been verified in other muscles, namely, rabbit and human.

The routine procedure adopted was the fixation of the desired muscle tissue, usually the leg muscles, in formalin (10 p.c.) for several days. At the end of this period the muscles were dissected free and any obvious non-muscular tissue discarded, the remainder being cut into small pieces and put into a "Waring blendor" with sufficient water to cover the blades (150 ml.). Blending was carried out for about three periods of eight minutes, the interval

between operations being used for cooling. It is desirable to keep the tissue as near to room temperature as possible. This treatment reduced the muscle to a suspension which exhibited strong flow birefringence. This suspension was then centrifuged and washed with distilled water in an angle centrifuge three or four times.

A drop of suitably dilute preparation was then transferred to a collodion film mounted on a grid. After drying, the specimens were usually stained with a so-called "electron stain," or shadowed after the method of Williams and Wyckoff (1946) using platinum or uranium as the shadowing agent so that the ratio of shadow length to object height was in all cases about 4:1. "Electron staining" involves attachment of a metal with a high atomic number, and hence a high electron scattering power, to the protein molecules to be examined. The "stains" employed were phosphotungstic and phosphomolybdic acids. The actual mechanism of attachment is not clear, but it is a most effective procedure for increasing the contrast of biological material. Phosphomolybdic acid was generally used in the investigation since it was found that muscle tissue often stains too deeply with phosphotungstic acid. The technique of digestion with enzymes which has often been used to advantage in the elucidation of biological structures was also employed in the present investigation.

With muscle, in which many of the protein constituents are water soluble or can be removed by water trituration, any procedure such as blending disorganizes the tissue to such an extent that the resulting components show little or no structural regularity. Treatment with formalin before blending seems in this case to be an ideal procedure. The small molecules penetrate thin strips of muscle easily and stabilize the structure by the formation of cross linkages (French and Edsall, 1945). After formalin fixation it is much more difficult to remove protein substance and the structure seems to be stabilized at the macromolecular level.

The electron microscope used in the investigation was an R.C.A. Model E.M.U. fitted with biassed electron gun, and objective aperture. The magnification of the instrument was calibrated using the method described by Farrant and Hodge (1948) in which the diameters of silica or glass fibres are determined by an interferometric method. The technique enables the magnification to be determined within one per cent.

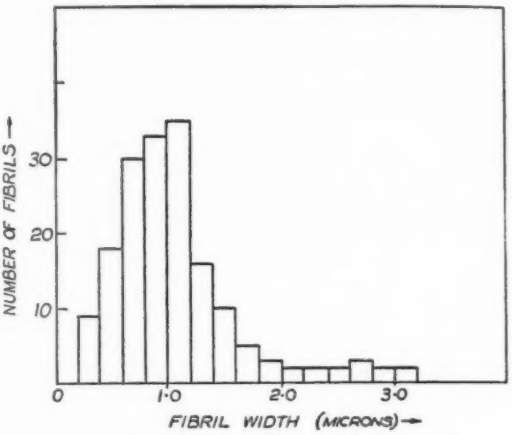
RESULTS.

The nomenclature to be adopted in presenting the results needs some explanation. The electron microscope (Hall, Jakus and Schmitt, 1946) has confirmed many of the earlier histological results and settled definitely some of the debated structural details. Consequently, it is possible in these cases to retain the generally adopted classical nomenclature without confusion, giving each term a greater significance in keeping with the greater structural detail revealed. Where it has not been possible to confirm the earlier beliefs some care must be taken in using a familiar term to describe a feature. Care will be taken here to define each term used and to conform as much as possible to accepted terminology. For further information on classical nomenclature reference may be made to the reviews of the subject by Meigs (1932) and Bremer and Weatherford (1944).

The type of dispersion produced by adequate blending of formalin-fixed material is shown in Fig. 1. The main constituents to be seen are numbers of ribbon-like structures exhibiting

clearly the known morphological features of striated muscle. The width of the individual ribbons is generally uniform throughout their length.

Size-frequency analysis shows (see diagram) that the width of these ribbons lies between 0·3 and 5μ, with a marked peak at about 1μ. This is evidence for a structural unit existing at this level of organization. It also suggests that these structures, produced by adequate blending, are probably identical with the myofibrils, sarcostyles, muscle columns or muscle fibrils observed by earlier workers. The term muscle fibril will be used in this text to identify these ribbon-like structures. The fact that prolonged blending does not appreciably alter the mean value of the fibril width is additional support for the thesis that the muscle fibril represents a structural unit at a level of organization intermediate between the gross muscle fibre and the protein filaments which constitute a substantial fraction of the muscle substance.



DISTRIBUTION OF WIDTHS OF 170 FIBRILS.
Distribution of widths of muscle fibrils in specimens from formalin-fixed muscle after blending.

The technique employed in the present study makes it very difficult to determine the manner in which the muscle fibre is built up from the muscle fibrils. A sectioning technique suitable for electron microscopical examination is the logical procedure to answer this question. Pease and Baker (1949), working with rat muscle, have produced convincing pictures of sections to show that the muscle fibre is made up of a series of thin-walled tubes about 1μ in diameter. The evidence to be presented indicates that the most probable interpretation of the flat ribbon-like components seen on the electron microscope screen is that they are collapsed tubes, the walls of which are composed of a single layer of protein filaments. Although there are other possible interpretations of some of the experimental observations, none of them fits all the available evidence.

The general histological features of the muscle fibrils as seen by the electron microscope have been described by Hall, Jakus and Schmitt (1946). These features can be seen from Figs. 3, 4, 7 and 8 where the lettering indicates the various bands and regions to be discussed subsequently. Examination of Figs. 1 and 2 show that the muscle fibrils exhibit a strong tendency to fracture at right angles to the fibre axis at either the Z band or the junction of the A and I bands. In one or two places a single fragment consisting of the region between Z bands can be seen (Fig. 2). The portion defined from Z band to Z band is known as a sarcomere. In other places fragments consisting of only the A band region can be seen. With

certain grinding techniques it is possible to produce fields consisting predominantly of single sarcomeres (e.g. Fig. 2). These observations indicate that the A band region is more robust than the I band region. This tendency to fracture at right angles to the fibre length has been frequently observed in the past. Shadowing techniques demonstrate clearly the ribbon-like nature of the fragments (Figs. 3 and 4).

Many of the fields examined contained a number of fine fibrils, which from their typical cross-banding, axial period and intraperiod structure, were identified as collagen. In addition to the collagen fibrils, a number of fibrils of similar width and axial period, but with a less well-defined cross-striation were observed. The latter fibrils have been tentatively identified as neurotubules, similar to those described by de Robertis and Schmitt (1948). A number of dense bodies of various shape and size are visible in Fig. 2. Similar bodies were occasionally seen apparently attached to sarcomeres in the Z band region by a small stalk. The significance of these structures is unknown, but they may represent mitochondria or similar cellular components.

In addition Fig. 25 shows a structure corresponding both dimensionally and in appearance with the nuclei seen in fresh muscle fibrils with the optical microscope. Some internal structure is evident but the density and thickness of the material prevent observation of fine details with 50 Kv. electrons.

Figs. 15 to 20 show the results of the action of bacterial proteolytic enzymes on blended formalin-fixed material after removal of excess formalin by repeated centrifugation and washing in distilled water. The Z and I band regions appear to have degenerated to amorphous protein aggregates. In contrast to this, the A band appears to have suffered relatively little, and is evidently much more resistant to these particular enzymes. The collagen fibrils are not obviously affected.

The use of enzymes has been of great value in the electron microscopical examination of fresh biological tissues, such as wool fibres (Mercer and Rees, 1946) and has also been successfully applied in the direct observation of morphological detail in formalin-fixed material, such as fowl spermatozoa (Grigg and Hodge, 1949). The preliminary results of enzymic action on fixed muscle indicate that the method may have a wide application in the electron microscopical examination of biological tissues, especially of those structures on which formalin fixation has a beneficial stabilizing effect. It should be noted, however, that the effect of enzymic action on formalin-fixed tissue is not necessarily the same as would be obtained on unfixed material.

The resistance to enzyme attack of the filaments in the A region is well illustrated in Fig. 15. This is a shadowed micrograph and the filamentous nature of the A band is clearly evident, yet in the I region this state of organization is no longer evident. The Z band has a diffuse appearance in contrast to the definite structure shown in Fig. 4. In the field of Fig. 15 can be seen a few filaments lying free, evidently having been frayed off the side of an A band. Several can be seen in the process of removal from the central sarcomere. Under these conditions the I band is essentially non-filamentous and easily disintegrated.

Protein Filaments of Muscle.

The fundamental constituent of the muscle fibril appears to be a protein filament showing an axial periodicity of about 400 Å (see Figs. 3, 4, 7 and 8).

As has been demonstrated by Draper and Hodge (1949), the fine cross-striation corresponds exactly with the distribution of mineral material within the sarcomere. This suggests that there exists, at these regularly spaced points

on the protein filament, reactive groups concerned with the binding or releasing of certain materials known to be involved in the mechanism of contraction.

Superimposed on the protein filaments are various aggregates of material, as indicated by the regions of high electron scattering designated as Z, A and M bands. This is the view put forward by Hall, Jakus and Schmitt (1946) and with which our results are in complete agreement.

Figs. 15 to 20 show various stages of enzymic degradation from fibrils such as those depicted in Figs. 3 and 8. Fig. 16 illustrates rather wide sarcomeres with all the I band material removed. Close examination of these isolated "A bands" shows that they appear to consist of two layers of protofibrils. This is consistent with the interpretation that they are collapsed single layer tubes. This view will be developed in subsequent sections. A point of interest is that the M band, while clearly visible in the mass of the sarcomere, is no longer distinguishable in those portions where the filaments have spread out and become separated.

Hall, Jakus and Schmitt (1946) demonstrated the continuity of the filaments from one sarcomere to the next. Our material also indicates that despite the tendency to fracture at the Z band region, the filaments have no evident discontinuity in this region. One border of Fig. 3 illustrates this point.

Hall, Jakus and Schmitt also demonstrated another striking feature associated with these filaments, namely, a knotted or beaded appearance, aligned transversely to produce a fine cross-striation with a period of about 400 Å, but considered that this was not sufficiently reproducible or regular to be designated as a periodic spacing in the nature of those found in collagen or clam muscle fibrils. We have found, however, that this fine striation is highly regular and extends throughout the sarcomere, the only variations in period, other than those irregularities resulting from the greater degree of collapse in the I band or stretching, being due to the state of contraction of the fibril. This is illustrated in Figs. 3 to 8. The fine cross-striation cannot generally be seen in the Z band because of the heavy overlay of material. It can be seen that the periodicity is manifested as a nodular surface configuration in shadowed pictures (e.g. Fig. 3) and as an increased electron scattering in stained specimens (e.g. Fig. 7). Many of the Figures illustrate the surface configuration very clearly.

The 400 Å period is due to a complex repeating unit analogous to that present in collagen fibrils. The repeating unit in the muscle filaments appears to consist of a linear array of several approximately isometric macromolecules, arranged in a definite order. This is illustrated in Fig. 8. Unfortunately, the resolution in our micrographs is not sufficient for a more detailed description of the structure within the repeating unit to be given, but it seems likely that three or more macromolecules, differing in structure from one another, are arranged longitudinally to form a repeating unit, approximately 400 × 150 Å.

Enzymic action does not appear to effect the periodicity in the A band (Figs. 15 and 17). The I band, however, shows no evidence of the periodicity, as a result of disorganization. In totally disorientated sarcomeres, as represented in Fig. 20, a periodic structure is just discernible in the individual filaments.

The axial period varies from 250 Å to 450 Å, and there is a fair correspondence with the sarcomere length. This suggests that the portion of a filament bounded by two adjacent fine striations is the ultimate functional unit of striated muscle. As far as can be determined, the periodicity is generally greater in the I than in the A band. However, it is difficult to obtain accurate estimates of the periodicity throughout a whole sarcomere, particularly in the

I band, where the greater initial degree of hydration results in more extensive disorganization during drying. Variations in stretching, most of which are probably the result of preparative methods, at present preclude any precise correlation of the axial period with the contractile processes. With improvements in technique it may be possible to collect sufficient data for a statistical treatment. This applies not only to the fine structure, but to the various relationships between the different regions, e.g. the relation of the A band length to sarcomere length.

Hall, Jakus and Schmitt (1946) used the term myosin filament to describe the structures under discussion. In view of the recent biochemical work summarized by Bailey (1944) and the work done by the Szent-Györgyi school (1947, 1948) it would seem advisable not to use the term myosin. This term is becoming recognized as part of a complex, actomyosin, believed by some workers to be the basic protein complex concerned with muscular contraction. The use of a word which in one field is becoming increasingly identified with a fraction of the protein complex responsible for muscular contraction may later lead to confusion. The use of the term acto-myosin filament as an alternative presupposes that the filaments are constituted of actomyosin alone and that actomyosin is the prime protein system concerned in muscular contraction. In view of the definite morphological nature of these structures the term *protofibril* would seem to be justified, especially as the term is non-committal regarding their ultimate chemical nature. On the other hand, the term filament has been used frequently by previous authors and hence it may be less confusing to use the term muscle protein filament.

The Z Band.

The general opinion held by the earlier histologists was that the Z band was a membrane running transversely across the muscle fibre and adherent to the sarcolemmal sheath at the periphery. The myofibrils or sarcostyles ran through this membrane. The chemical nature of the Z band has also aroused interest. It was believed to be collagenous in nature, but the electron microscopical studies of Hall, Jakus and Schmitt (1946) dispelled this idea. In electron microscopical preparations the Z band is a region of great electron scattering. Close inspection of Figs. 3 to 6 shows that the surface of the Z band is irregular and no two Z band surfaces are exactly the same. This is consistent with an irregular cleavage of a not too highly organized protein mass. The Z band region is quite often found to be distorted, as if it were in part a structure of loose texture lying superficially on the muscle protein filaments, this portion being relatively easily distorted.

There is, however, some structure demonstrable in the Z band. Fig. 13 shows a specimen where the Z band can be seen protruding laterally from the edge of the muscle fibril. This protrusion was noted by earlier workers; Meigs (1932) commented on this point and published

a photomicrograph illustrating the matter in frog muscle: Fig. 13b shows an organization of the Z band into a series of fine sub-bands running across the muscle fibril. There appear to be two lateral sub-bands about 450 Å wide, and, in between these, three symmetrically placed bands about 100 Å in diameter. These sub-bands show no signs of an axial periodicity and are presumably, therefore, not to be identified as collagenous.

Apparently in this particular case the covering of dense superficial material had been removed. This is a rare occurrence and indicates that the Z band as a structure is closely knit internally. This would be expected if the Z band apart from any possible metabolic functions, is primarily a structural feature.

This latter interpretation of the Z region is supported by Figs. 21, 22 and 23 (particularly in Fig. 22) which show remnants of the sarcolemmal sheath. Running across the sheet of complex material can be seen a series of parallel lines of higher density. Fig. 22 is a portion of a 1,000 μ length of what must have been a complete sheath opened out. The parallel lines are very striking. The axial spacing of these lines, together with the older observation of the adherence of the Z region to the sarcolemmal sheath (Cajal 1933, Meigs 1932) supports the belief that these lines in Figs. 21 to 23 represent regions where the Z bands adhere to the sarcolemmal sheath. After mechanical disruption, portions of these structures are left adhering to the collagenous sheath. Not all the fibrils we examined can have been adherent to the sarcolemmal sheath but the available evidence indicates that more internally situated fibrils will be adherent to one another at the Z band region. The structure of the sarcolemmal sheath will be described in more detail in a later section.

Using a layer-stripping method, Reed and Rudall (1948) suggested that the Z and M bands appeared as depressions. We are not, however, in agreement with this tentative identification of the A and I bands, as indicated in Figs. 2 and 3 of their paper. These micrographs were obtained by layer-stripping from dehydrated formalin-fixed muscle. Close examination of the distribution of height in these two micrographs, and particularly of the slight elevation in the centre of the alleged I band in their Fig. 3 shows a close correspondence with the height distribution in Fig. 3 (this paper), but in reverse. That is, heights correspond to depressions and vice versa. Further support for our contention is given by the anomalous ratio existing between A and I band lengths in Fig. 3 of Reed and Rudall's paper. In our experience, it seems unlikely that the ratio of I and A band width could be about 2:1 in such a fibril from frog muscle, the sarcomere length of which indicates that it is a relaxed fibril. Moreover, if the layer-stripping method employed by these workers is examined in detail, it becomes clear that the surfaces depicted in Figs. 2 and 3 of their paper are internally facing with respect to the muscle fibre. In our opinion, these surfaces represent negative replicas of the filamentous sheets. On dehydration, these units will have a distribution of height similar to that in Fig. 3 (this paper) and will leave a negative impression in the amorphous material which permeates all spaces between the units. On the basis of these considerations, we feel that the designation of bands expressed by Reed and Rudall should be reversed. If this is done, the distribution of height along the fibre axis corresponds exactly in reverse with that present in Fig. 3 of the present paper.

The M Band.

The M band differs in some respects from the Z band. The filaments of the sarcomere can nearly always be observed to run through the band (e.g. Figs. 3 and 4). This very rarely occurs in the Z region and indicates that there is little or no overlay of material in the M region. That there is material other than the filaments present in this region is indi-

cated by the increased electron scattering and by the shadow length. In fact the M band is generally almost as high as the Z band.

The presence of the M band substance between the muscle filaments seems to preserve the alignment of the protofibrils in a remarkable way. Fig. 10 shows the regularity of the filaments just before entering the M band. It is as if the M band is here acting as a spacer, so much so that in many places, owing to loss of water or other material and because of the spacing action of the M band, the filaments have been unable to draw together, and so "holes" have appeared close to the band. The diameter of the filaments in this region is about 160 Å. In places along the M band in Fig. 10, if the "holes" are closely examined, the impression is given that the muscle filaments are in two layers. Due to the slight twisting of the filament sheet the lower layer has come into view. This seems to support the previously mentioned concept of the fibrils as collapsed thin-walled tubes.

In specimens that have been subjected to mild proteolytic enzyme action (Figs. 15 to 20) some further features become evident. Previously it was noted that the Z band under these conditions was, in company with the neighbouring I band, largely disorganized. Such complete disorganization does not occur in the M band, which is well defined in Figs. 15 and 17 where the filaments are prominently displayed throughout the band. The M band can still be seen in sarcomeres such as are shown in Fig. 16. Here a fragment of a muscle fibril is in the process of disintegration to something like Fig. 20. As long as the filaments are aligned closely side by side the M band stands out. In the regions where single isolated filaments exist or the alignment is grossly disturbed, the M band is no longer evident, either in stained or shadowed specimens. In Figs. 16 and 19 it can be seen that, despite the ragged appearance of the ends of the filaments, they are still aligned side by side in the M region with surprising regularity, considering the degraded state of the rest of the sarcomere. Evidently, under these conditions the M band substance is the last barrier to total disruption of the sarcomere. Once this substance is interfered with a mass of filaments results, as shown in Fig. 20.

From the above considerations it might appear that the whole of the structural rigidity displayed by the M band as compared with that of the Z band is a consequence of a fundamental structural differentiation. However, it would be unwise to disregard the obvious stabilizing effect that the A substance confers on the material in its immediate vicinity. It is evident from a consideration of the distribution of structural height after desiccation, that the A substance, which may be present at any region within the sarcomere, its distribution depending on the state of contraction, is relatively low in water content and tends to prevent collapse of the tissue during drying. In addition, the A substance contains globular protein material as well as mineral constituents. It seems likely that on formalin fixation numerous cross-linkages are formed between the filaments and the globular proteins, the net result being the creation of a relatively stable structure, both as regards dehydration and enzymic attack. Thus the distribution of height in a desiccated muscle fibril (cf. shadowed micrographs) appears to be related to the degree of hydration of the material associated with the filaments prior to dehydration. It will be clear from a consideration of Figs. 15 to 19 that the resistance of the filaments to enzymic attack is also directly related to the distribution of A substance.

The H Band.

The Z and M bands are the fixed points in a muscle fibril. They may always be distinguished irrespective of the state of contraction of the muscle fibril after fixation. They are, also, within the limits indicated, relatively robust structures. The remaining features of the sarcomere are less constant and appear to depend on the state of contraction of the muscle when fixed. The previous history of the fibre before fixing is another factor to be considered. The state of contraction of a fibre is not easily controlled, particularly as the fixative usually acts as a stimulant. The difficulty is to obtain a muscle fibre that is definitely in a relaxed state.

The H band, as defined by optical microscopical methods, was a relatively clear region bisecting the A band and in turn bisected by the M band (Figs. 4 and 8). Between the M band and the beginning of the A region proper the electron microscope reveals two fine dark sub-bands (Fig. 8). Hall, Jakus and Schmitt (1946) commented that these sub-bands were not constant, and beyond adding that they were not uncommonly found in our material, we can only endorse their finding. The significance of these sub-bands is not known, but they appear to arise from an enhancement of the normal fine striation. Their greater spacing as compared with the normal axial period, may be due to stretching of the H band, since the latter band is more frequently found in fibrils from muscles which have been stretched during fixation. Fig. 8 shows these sub-bands clearly, while Fig. 4 shows that their greater density is also reflected as a difference in height, suggesting that there is here localized a greater amount of mineral material than that present in the normal fine striation. This has been confirmed by micro-incineration within the electron microscope (Draper and Hodge, 1949). Occasionally, only one of these sub-bands is visible, that is to say one on either side of the M band. They are most clearly seen in those fibrils possessing H bands. Fig. 9 shows a rather wide H band and two sub-bands can just be made out. As was pointed out by Hall, Jakus and Schmitt (1946) the presence of an H band is dependent on the state of contraction of the fibril and the condition under which fixation was carried out—i.e. whether isotonic or isometric, the latter condition being conducive to the formation of the H band. This point will be discussed further in the section on contraction.

The A Band.

The A band or anisotropic region is generally taken to be the whole region between two successive I or isotropic regions; i.e. the dense region bisected by the M band and the H band, when present. Hall, Jakus and Schmitt (1946) have made it clear that the presence of protein filaments aligned parallel is sufficient to explain the birefringence of the region, and suggested that the anisotropy is compensated in the I band by some other substance. This suggestion has been confirmed by the extraction of a negatively birefringent material by Matoltsy and Gerendas (1947). It is also clear that the high density of the A band is due to the presence of the A substance, which contains the bulk of the mineral ash (Macallum, 1905; Scott and Packer, 1939). The presence of the A substance also accounts for the distribution of height within the sarcomeres of desiccated fibrils as seen in shadowed electron micrographs

(Fig. 3). In addition, the A substance is known to migrate within the sarcomere during contraction, as described by Jordan (1933) and confirmed by Hall, Jakus and Schmitt. Our results are also in agreement with this finding. However, the changes in distribution of birefringence within the sarcomere during contraction have not yet been adequately described. Thus, anisotropy of the A band might equally well be due to the A substance as to the birefringence of the filaments. In this event one would merely suppose that the state of affairs obtaining in the I band, namely, compensation of the filament form birefringence by a negatively birefringent material, is applicable throughout the length of the sarcomere. If this is the case, and the anisotropy of the A region is due to the A substance one would expect an exact correspondence of the distributions of A substance and anisotropy within the sarcomere.

Fig. 3 shows that next to the Z, the A band is the thickest region of the dehydrated fibril. Whether such differences in height exist in the living tissue is a controversial point, but there can be no doubt that much of the difference is due to the partial collapse of those regions with the greatest *in vivo* degree of hydration, viz. I and H bands. Figs. 3 to 6 make it clear that the H band could arise as a result of the migration of A substance away from the M band.

The distal border of the A band, or the A–I junction is usually well-defined and abrupt. In view of the fact that there is no evidence for a confining membrane, one must suppose that the A substance is limited by forces of interaction with the material present in the fine cross-striations. Thus, one might envisage the migration of the A substance as proceeding only in discrete steps corresponding to the axial period of the fine cross-striations, the flow of A substance being initiated or checked by some as yet unknown processes involving reactive centres contained in these fine striations.

In micrographs where the fine cross-striations are well-defined, particularly those of shadowed specimens, it will be seen that the periodicity is obvious in both the A and I bands, although less so in the latter, due to partial collapse of the structure. Thus, it is clear that the relative thickness of the A band is not simply due to an overlay of material. The A substance cannot reside wholly in between the filaments because there is no significant difference between the width of the A or I regions. This means that the bulk of the A substance must reside on the inner surface of the tube wall. In shadowed micrographs the surface configuration of the A band is always the same. This indicates that the top and bottom surfaces of the fibril are identical, and in between these surfaces exists the A substance. This is what one would expect if in life the fibril is tubular, with the A substance arranged on the inner wall of the tube. On dehydration and resultant collapse of the tube the filamentous wall will retain its sheet-like nature due to lateral association of the filaments, and the form of the collapsed tube will depend on the removal or otherwise of the contents, and other factors. It is hoped that future experiments with enzymes will clarify the situation and enable us to be less speculative about the muscle fibril and its relationship to the muscle fibre.

The I Band.

The electron microscope has shown that the I band is structurally the least complex region of the sarcomere. In the preceding section it was suggested that in life it is more highly hydrated than any other region. It consists essentially

of protein filaments aligned parallel and arranged in "lamellar" fashion as already described. In view of this, the term isotropic would seem to be anomalous, but as has been stated, Hall, Jakus and Schmitt (1946) suggested that the low birefringence of the I band is due to almost complete compensation by other components. This was apparently confirmed by the work of Matoltsy and Gerendas (1947), who demonstrated that it is possible by suitable extraction methods to restore the form birefringence of the I band.

Of late there has been some controversy concerning the distribution of nucleic acids within the sarcomere. Many of the apparently contradictory findings are no doubt due to the limited resolution obtainable with the light microscope even when using ultraviolet illumination. The electron microscope results make it clear that the bulk of solid material within the sarcomere is concentrated in the Z, M and A bands, the I band containing little but the protein filaments.

Apart from the features already discussed, the only other differentiation occurs near the A–I junction and takes the form of a variable accumulation of substance in the usual cross band form. This may appear as a pronounced double band (Fig. 14) or as a much less obvious band (Figs. 3, 4, 7 and 8). In many cases, as when the A substance is uniformly distributed throughout the sarcomere or localized in the Z region, this feature cannot be demonstrated. This is in accord with the findings of Hall, Jakus and Schmitt in the frog and rabbit. They used the term N band in accord with earlier histological usage. In stained preparations the N band appears as a slight increase in image density (Figs. 9 and 14). In our material some semblance of N bands can be seen in about 20 per cent. of the fibrils.

The I band, and any morphological features associated with it, are more easily obliterated than the A band features. This applies to the fine cross-striation as well as to the N band; (the Z band is, strictly speaking, a part of the I band but is not being considered here). If the suggestion that this region is more hydrated than the A band is valid, then by the loss of this water it is feasible that a relatively greater variability could be introduced, there being less protection from mechanical and chemical agents.

In shadowed micrographs (Figs. 3 and 4) the N band appears as one or two ridges raised above the level of the I band. Thus here, too, we have a localized concentration of dense material, the state of affairs being similar to that obtaining in the A band, where the interstitial material prevents collapse on dehydration. This lends support to our belief that the distribution of height in dehydrated fibrils corresponds qualitatively with the density distribution, as reflected in the electron scattering power.

Morphological Features of Contraction.

In our present state of knowledge this is a difficult aspect of the study upon which to comment. In using the term contraction we are referring to a physicochemical process whereby a protein system is enabled to perform mechanical work or exert a mechanical force. In the latter case the overall length of the macroscopic muscle need not change. This is the isometric contraction of the physiologist, as opposed to isotonic contraction, in which the muscle mass shortens and performs external work. If the macroscopic behaviour of the

muscle is representative of its molecular behaviour, this represents a very interesting aspect of the problem.

Whether the contraction be isometric or isotonic, the physiological mechanism for producing and maintaining the change appears to be the same. The sustained tension in a muscle is not the result of a distortion of a particular system—in this case the muscle fibre—for the duration of the tension. On the contrary, contraction of a whole muscle is an integrated response of a mass of fibres, each one contracting for about a thousandth of a second and then relaxing, repeating this performance from one to forty or so times a second. The greater the tension the greater the rate at which each unit contracts (*vide* Denny-Brown and Pennybacker, 1939). Each fibre is linked to many other fibres (50–200) to form a group known as a motor unit, the components of which are activated and relax simultaneously. The net contraction of a muscle is caused by the asynchronous combination of tens of thousands of motor units, each unit exerting a twitch force of the order of grams weight each time it is excited. In addition the initial length of a fibre, within physiological limits, has an influence on the resultant force of contraction. The exact nature of the response has been reviewed by Fenn (1947).

Although contraction of a muscle appears to be a simple phenomenon, nevertheless, as can be seen from the above brief comment, the actual process of implementation of the mechanical effect is far from straightforward. It could be argued that the contraction, i.e. the resultant movement *possible*, is not a necessary property, because movement of the system is not an inevitable conclusion once the process has been activated. On such a basis one could imagine the process to be the momentary induction of an increased attraction between molecular groupings in a longitudinally orientated system. The molecular matrix in which this system exists is capable of reversible mechanical distortion and thus, if the resistance opposing the increased molecular attraction is not too great, the system can shorten. If the external resistance is too great then no shortening need occur. In both cases a force has been applied to an external system, the only difference being whether or not external work has been done. In the former case, the external work done represents but a small fraction of the total energy exchange.

Besides the above-mentioned normal physiological mechanism there is a state of muscle known as contracture, in which the muscle exists as a whole in a shortened state. This is induced by various non-physiological procedures such as the application of strong electric fields, heat and certain drugs (Gasser, 1930). Thus, in discussing the so-called contraction of muscle, one should be very careful to define exactly in what way the term is to be used. Though the molecular mechanisms of contracture and contraction may be essentially similar, this is not yet proved. It is possible that many of the states of supposed contraction are in reality states of contracture.

In the electron micrographs one can observe many overall features that may or may not be a reflection of the state of contraction or contracture of the muscle fibril at the moment of fixation. Just how these changes may be related to physiological contraction it is not possible to state at this juncture. The process of preparation of a living fibre for examination in the electron microscope is very complex, and many of the stages are known to be excitatory in a non-physiological manner. For example, the action of formalin is non-physiological, and, moreover, a further complication is introduced in that the outside fibres receive a greater amount in a shorter time and this may well have a different effect on the muscle from the centrally located fibres which have a much lower rate of application by diffusion.

From classical histology comes the thesis that the migration of the A band substance towards the Z band accompanies contraction. Nothing in the electron microscopical evidence to date is at variance with this idea, and, apart from the difficulty in determining the exact degree of contraction of any given fibril, the electron microscopical evidence can be interpreted as confirmation of this earlier concept. Hall, Jakus and Schmitt suggested that the widening H band as in Figs. 4, 8 and 9 is, apparently, associated with isometric contraction, while in isotonic contraction a complete migration occurs as in Fig. 12. In these the supposition is that the A substance has migrated from the M band into the Z band region producing a more pronounced Z band region (contraction band), the remainder of the sarcomere now being of uniformly low density. If the time of normal contraction is taken to be 0·002 sec., then the rate of migration of the A substance must be about 0·2 mm. per sec., a value of the correct order for a diffusion or electrokinetic process. Figs. 5 and 11 show two fibrils in a mild state of contraction, as judged by sarcomere length, and with a relatively uniform distribution of A substance. The fine transverse striation is very clearly defined in Fig. 5 and the Z bands are easily differentiated from the M bands by their irregular appearance. Both fibrils, presumably, represent mild stages of contraction intermediate between those represented in Figs. 3 and 12. Such fibrils are not uncommon in our preparations.

It seems reasonable, because of the lack of distortion and the high degree of order prevailing, that Fig. 3 represents a fibril in a state of relaxation. The fibril depicted in Fig. 12 has quite evidently undergone strong contraction, and complete "reversal of striation" has occurred. The other types of sarcomere shown in the various figures, presumably, all represent stages of contraction intermediate between these two, the distribution of the A substance being determined in each case by the conditions prevailing at the time of fixation, such as stress, degree of stimulation and whether contraction is isometric or isotonic.

In spite of the errors introduced by fortuitous stretching of the fibrils during the process of mechanical dispersion, there is a distinct correlation between the sarcomere length and the type of distribution of A substance within the sarcomere. Although it is not yet possible to describe in detail the changes in distribution of A substance during contraction, our results confirm the "reversal of striation" phenomenon.

The Sarcolemmal Sheath.

This structure has been the subject of more speculation than any other in the field of muscle structure. Estimates of its thickness have varied within wide limits (Barer, 1948) and the electrolyte balance between its interior and

exterior surfaces has been intensively studied with very little result. The electron microscope has shown it to be in actual fact a complex structure possessing at least two differentiated components. Reed and Rudall (1948) using a layer-stripping technique, demonstrated the presence of (a) a thin membrane, structureless save for numbers of regular discrete corpuscles resting on its surface; (b) a layer of collagen fibrils, running helically around the muscle fibre.

Further evidence for the presence of a thin structureless membrane was obtained by Jones and Barer (1948), who obtained specimens by teasing fresh muscle in order to stimulate the formation of retraction clots. Their micrographs also showed the discrete corpuscles—apparently randomly distributed.

We have often observed large expanses of sarcolemmal material in our preparations. Figs. 21 and 22 were taken from a fragment approximately 1,000 μ long and from its dimensions and shape it must represent practically a complete section of a sheath rolled out. Running at right angles to the fibre axis can be seen the Z band remnants previously referred to. It will be noticed that short segments, the lengths of which are commensurate with the average fibril width are displaced slightly in the direction of the fibre axis. A corresponding displacement can be traced throughout all the Z bands in the sheet. If the muscle fibre consists of a large number of close packed units about 1μ in diameter these discontinuities in the Z band could represent a slight displacement between adjacent units. This is probably due to an inherent fault in the lateral alignment of the fibrils during growth. A displacement of the individual fibrils within the muscle fibril has also been demonstrated by Reed and Rudall (1948) using a layer-stripping method.

In Figs. 21 and 22 folds of the membrane can be seen and a comparison can be made between the two surfaces. Fig. 21 shows a larger fold and from these illustrations it can be seen that the two surfaces differ markedly. On one there is a complex array of collagen fibrils arranged in a network of whorls. The main trends of this distribution can be seen from Fig. 23, although there has been quite extensive disorganization during specimen preparation. On the other surface there is the amorphous Z band material superimposed on an apparently amorphous flat sheet. This is evidently the internal surface; and the surface with the complex network of collagen, the external surface of this sheet. Fig. 24A shows the internal surface in greater detail. It can be seen that the Z band remnant appears to be structurally continuous with the rest of the membrane which consists of two main components. The first is a network of very fine fibrils (*ca* 100 Å diameter) (Fig. 24B) which do not appear to be collagenous as there is no evidence of the characteristic spacing. Surrounding this network is a matrix material which can be seen to be in many places in regular granules. In both the fibrils and the granules there appears to be some organization which suggests a helical alignment around the whole muscle fibre (Fig. 24A). In the same way on the external surface (Fig. 23) collagen can be seen to be arranged in bundles which have a general organization at about 45° and 135° to the muscle fibre axis.

Fig. 23 shows, in addition to the diagonal arrays of collagen, a series of transverse parallel arrays of fibres situated at about 2 μ apart. This is the same distance apart as the Z band remnants on the inner surface and suggests that the collagen is here arranged to reinforce that portion of the membrane associated with the Z band. The general arrangement of the collagen suggests that this may be the biological device by means of which is obtained an elastic membrane, capable of giving structural support to the muscle fibre and yet

capable of transmitting tension to the tendon. As is known, collagen is relatively inextensible when the fibres are arranged in parallel bundles as in tendon.

From the work of Reed and Rudall (1948), Barer (1947), Jones and Barer (1948) and the present observations, it can be seen that the sarcolemmal sheath is in reality a very complex structure. We suggest, tentatively, three main constituents. There is the aggregate of fine fibrils embedded in a matrix material which is apparently continuous with the Z bands of the muscle fibrils. Surrounding this is a helical and annular array of collagen. In the examples shown (Figs. 21 and 22) the above complex is of the order of 400 Å thick. As we do not know how much collagen has been removed in the preparation, nor the shrinkage due to dehydration, this thickness cannot help very much in the assessment of sheath thickness in the living muscle. The third component is the interesting spotted membrane demonstrated convincingly by Jones and Barer (1947) and Reed and Rudall (1948). The ease with which retraction clots may be formed in muscle (Barer, 1947) indicates that this membrane from material with induced retraction clots is loosely attached to the underlying complex. In addition, their work indicates that it should be the outermost component of the complex sarcolemmal sheath.

SUMMARY.

The striated muscle of the toad, *Bufo marinus*, has been studied using the electron microscope (EM). Preparative techniques employed included formalin fixation, homogenization with the "Waring blendor", enzymic digestion, "electron staining" and metallic shadow-casting with platinum or uranium. Toad muscle was found to be highly suitable for electron microscopical investigation and many features of the ultrastructure have been revealed. Preliminary studies on the striated muscles of other animals indicate that the results described here are for the most part also applicable to them.

The muscle fibre consists of a number of sub-units or muscle fibrils, arranged in almost perfect lateral and longitudinal alignment, and surrounded by a complex sarcolemmal sheath with several well-defined components. The innermost layer of this sheath consists of a granular matrix material in which are embedded many fine interlacing fibrils, about 100 Å in diameter, which do not appear to be collagenous. It seems likely that they represent a hitherto undescribed structural component of animal tissues. The innermost layer of the sheath is either firmly attached to, or structurally continuous with, the Z bands of the muscle fibrils. Immediately surrounding this layer is a series of collagen fibril bundles, arranged in annular fashion about the muscle fibre and reinforcing the membrane at the "lines of attachment" of the Z bands. Surrounding the inner sheath are several collagenous layers, the fibrils of which are aligned parallel within each layer and wound helically about the fibre. The

direction of the helix is reversed with each consecutive layer. The outermost component of the sarcolemmal sheath is a thin loosely-attached membrane, apparently structureless save for a number of granules, and may be isolated by inducing the formation of retraction clots.

The muscle fibrils which comprise the bulk of the muscle fibre appear to be thin-walled tubes, the walls consisting of a single layer of muscle protein filaments or protofibrils, each about 160 Å in diameter, which are arranged with almost perfect lateral and longitudinal alignment throughout the length of the fibril. The tube-like muscle fibrils, which appear to be identical with the myofibrils of the early histologists, appear as thin ribbons in electron microscopical preparations, as a result of the necessary dehydration before examination. Size-frequency analysis shows that the widths of the ribbons varies from 0·3–3·0μ with a pronounced maximum at about 1·0μ. This represents strong evidence for a definite morphological unit at this level of organization.

The muscle fibrils display the usual histological features, viz., Z, M, A, I, H and N bands, and, in addition, a fine cross-striation, the axial period being in the range 250–450 Å, depending on the state of contraction of the fibril. The regularity of this cross-striation, which is also observable in individual filaments, leaves little doubt that it must be regarded as a repeating pattern analogous to that observed in other fibrous proteins, such as collagen, "neurotubules" and keratin. The fine structure of the various bands has been described in detail.

The changes in the distribution of the A substance within the sarcomere during contraction have been described and discussed. The electron microscopical results confirm that there is a "reversal of striation" in strong contraction, and that this is due to the migration of the A substance to the Z bands. Intermediate stages of contraction appear to be characterized by definite "band patterns" within the sarcomere. The observation that there is a rough correlation between the axial period in the muscle protein filaments and the state of contraction of the fibril, as estimated by sarcomere length, suggests that the ultimate contractile unit in striated muscle is that portion of a single filament contained in one axial period (i.e. between two adjacent fine cross-striations).

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KEY TO ABBREVIATIONS USED IN ELECTRON MICROGRAPHS.

Z	Z band or Telophragma.	mf.	Muscle protein filaments or protofibrils.
M	M band or Mesophragma.	MF	Muscle fibrils or myofibrils.
A	A or Anisotropic band.	S	sarcomere or inokomma.
I	I or Isotropic band.	cf	collagen fibrils.
H	H band.	Hsb	H sub-bands.
N	N band.	C	Contraction bands.
Zbr.	Z band remnants.	mn	Muscle nuclei.

EXPLANATION OF ELECTRON MICROGRAPHS.

Fig. 1. Typical low-magnification field from formalin-fixed and blended muscle, air-dried and shadow-cast with platinum. Note different types of sarcomeres and vertical profiles as shown by shadow outlines. (× 2,600).

Fig. 2. Typical low-magnification field from muscle after formalin fixation and grinding, air-dried and stained with phosphotungstic acid. Note short fibrils, single sarcomeres, collagen fibrils and dense bodies. (× 7,750).

Fig. 3. Relaxed muscle fibril, shadow-cast with platinum. Note fine cross-striation throughout the sarcomere, difference between Z and M bands, and vertical profile as shown in shadow outline. (× 35,400).

Fig. 4. Relaxed and stretched muscle fibril, shadow-cast with platinum, showing wide H band. Note filaments running through M band but not through Z band. (× 35,200).

Fig. 5. Semi-contracted muscle fibril, shadow-cast with platinum. Note regularity of the fine cross-striation extending throughout the sarcomere. (× 35,400).

Fig. 6. Muscle fibril with wide A band, shadow-cast with platinum. Direction of shadowing emphasizes cross-striation. (× 42,700).

Fig. 7. Muscle fibril in similar state to that shown in Fig. 3, stained with phosphomolybdic acid. Note filaments and fine cross-striations throughout the sarcomere. (× 64,000).

Fig. 8. Muscle fibril showing H band with four dark sub-bands. Stained with phosphomolybdic acid. (× 39,900).

Fig. 9. Muscle fibrils showing wide H band, stained with phosphomolybdic acid. (× 29,400).

Fig. 10. Portion of fibril stained with phosphotungstic acid showing lateral alignment of filaments in the M band region, and four fine sub-bands in the H band. (× 48,600).

Fig. 11. Semi-contracted muscle fibril with A substance extending almost throughout the sarcomere, showing fine cross striations and two dark sub-bands on either side of the M band. Stained with phosphomolybdic acid. (× 41.700).

Fig. 12. Strongly contracted muscle fibrils, showing complete reversal of striation and formation of contraction bands. Stained with phosphomolybdic acid. (× 29,400).

Fig. 13. Muscle fibril with protruding Z bands. (× 29,400).

Fig. 13b shows sub-banding in more detail. Stained with phosphomolybdic acid. (× 88,000).

Fig. 14. Muscle fibril, stained with phosphomolybdic acid, showing doubling of N bands. (× 29,400).

Fig. 15. Muscle fibril from sample exposed to bacterial enzymes, shadow-cast with platinum. Note the extreme thinness of the muscle fibril, the individual muscle filaments and collagen fibrils in the background, and the degradation of I and Z bands. (× 28,400).

Fig. 16. Group of isolated "A bands" from a sample after exposure to bacterial enzymes. The I and Z regions of the sarcomeres have been removed entirely. Note individual filaments fraying from sarcomere edges and in background. Stained with phosphomolybdic acid. (× 22,200).

Fig. 17. Group of muscle fibrils from a sample treated with bacterial enzymes, showing greater degree of degradation of I and Z bands than in Fig. 15. Note high degree of regularity still present in A and M bands. Shadow-cast with platinum. (× 22,000).

Fig. 18. Group of muscle fibrils from a sample treated with bacterial enzymes, showing extensive disorganization of I and Z bands. Stained with phosphomolybdic acid. (× 25,000).

Fig. 19. Frayed end of muscle fibril from a sample treated with bacterial enzymes. Note individual filaments and remnants of M bands. Stained with phosphomolybdic acid. (× 28,200).

Fig. 20. Advanced stage of degradation after treatment with bacterial enzymes. Individual filaments with almost complete lack of the original alignment. Note periodic structure in the filaments, corresponding to the fine cross-striation in intact fibrils. Stained with phosphomolybdic acid. (× 45,600).

Fig. 21. Portion of sarcolemmal sheath from formalin-fixed and blended muscle. Folds show both surfaces of the sheath. Note parallel Z band remnants with marked displacements and layers of collagen fibrils. Shadow-cast with uranium. (× 4,580).

Fig. 22. Portion of sarcolemmal sheath from same specimen as Fig. 21, showing internal surface with respect to muscle fibre. Note parallel Z band remnants running normally to the fibre axis, and slight displacements resulting from misalignment of the muscle fibrils. Note also the collagen fibrils beneath sarcolemmal sheath, i.e. on external surface with respect to the muscle fibre. Shadow-cast with uranium. (× 3,790).

Fig. 23. Portion of sarcolemmal sheath at higher magnification than Fig. 22 from specimen treated with bacterial enzymes and shadow-cast with platinum, showing external surface of sheath. Note bundles of collagen fibrils running in a series of parallel lines across the sheath and corresponding in position with the Z band remnants seen in Fig. 22. Note also the diagonal trend of most of the remaining collagen. The rod-like bodies are bacteria. (× 6,000).

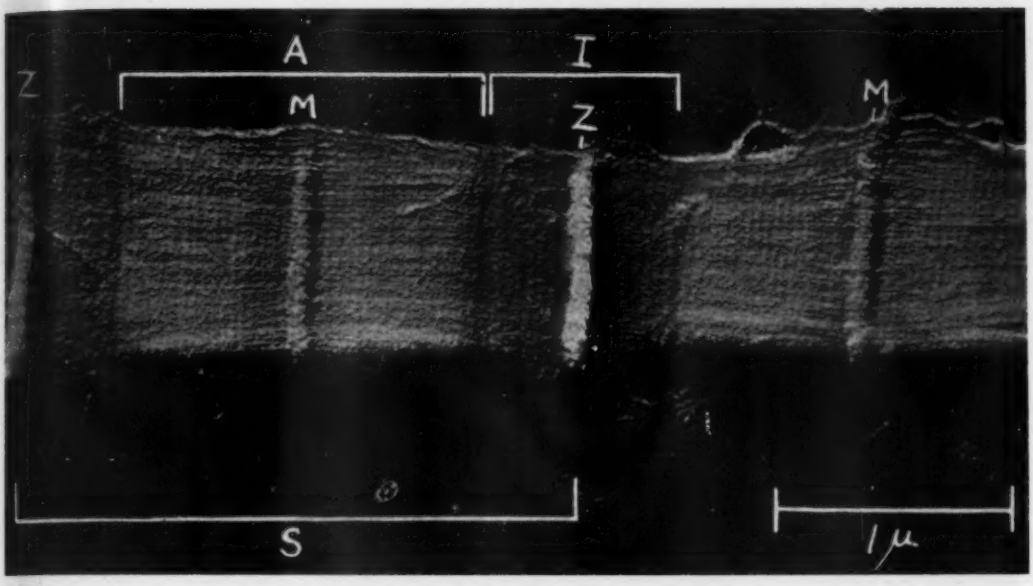
Fig. 24a. Portion of the sarcolemmal sheath shown in Fig. 22 taken at higher electron-optical magnification to show details of internal surface. Note Z band remnant, granules and fine fibrils arranged at about 45° to fibre-axis. Shadow-cast with uranium. (× 48,400).

Fig. 24b. Another portion of the sarcolemmal sheath in Fig. 22, showing torn edge of sheath. Note diagonal arrangement of collagen fibrils and their parallel alignment, also alteration of direction in collagen layers and apparent alignment of the small granules with the collagen fibrils. Shadow-cast with uranium. (× 27,000).

Fig. 25. Muscle nuclei in a specimen treated with bacterial enzymes. Note the vague internal structure. Shadow-cast with platinum. The small rod-like objects are bacteria. (× 6,000).

Fig. 1.

Fig. 2.



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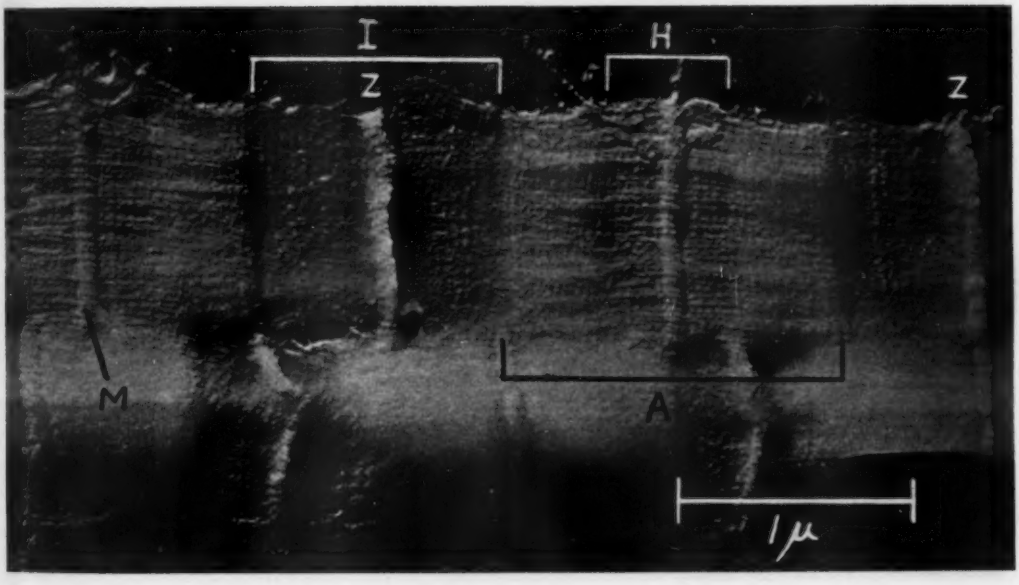

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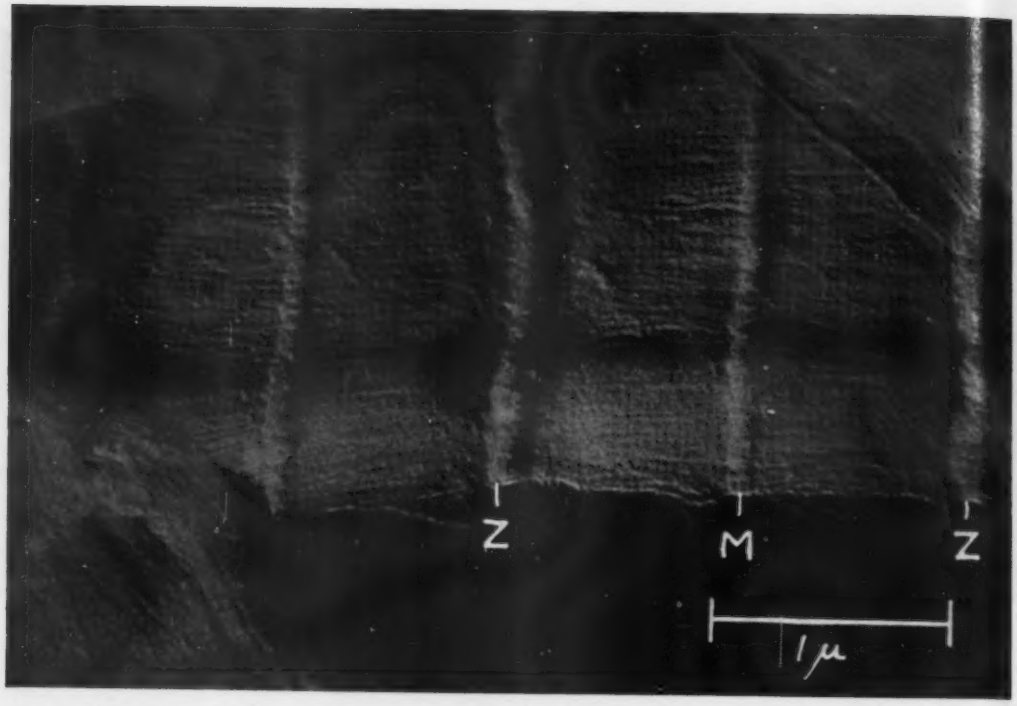

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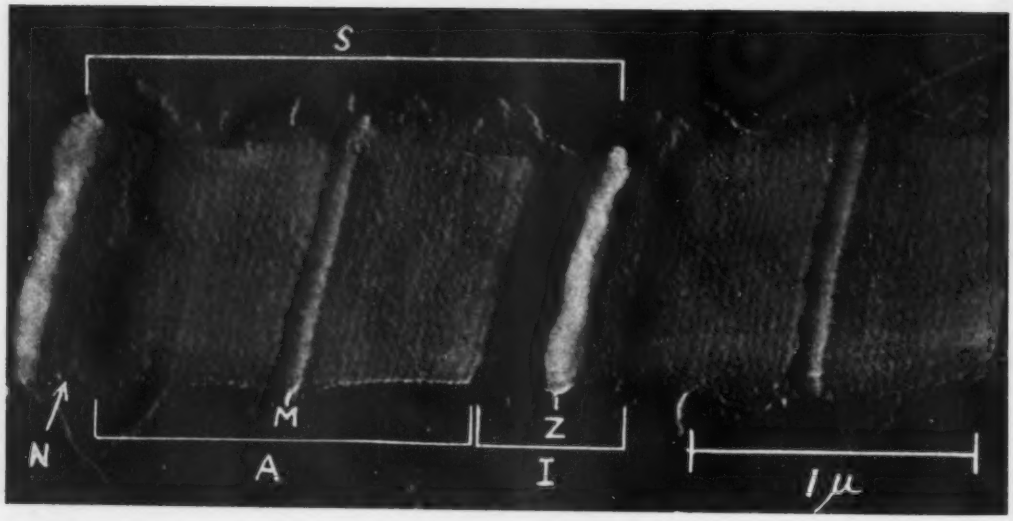

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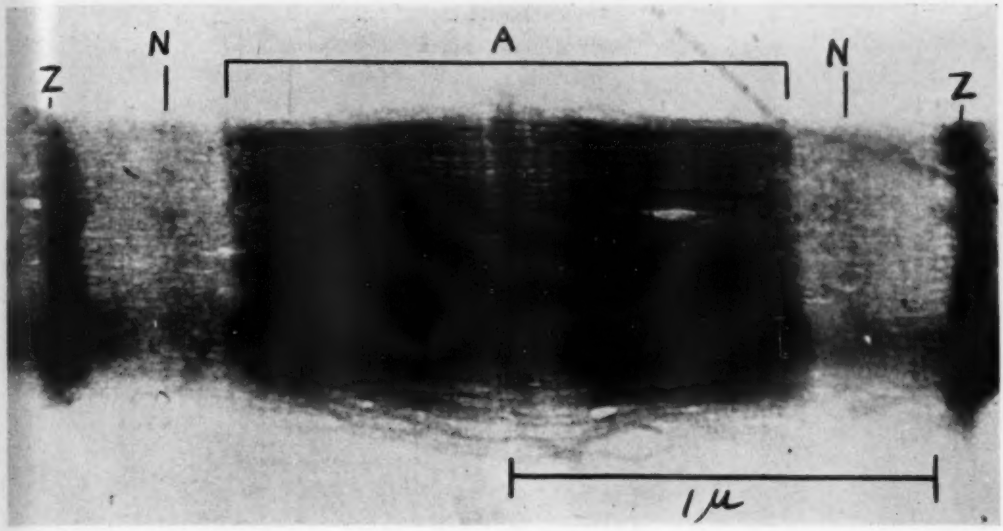

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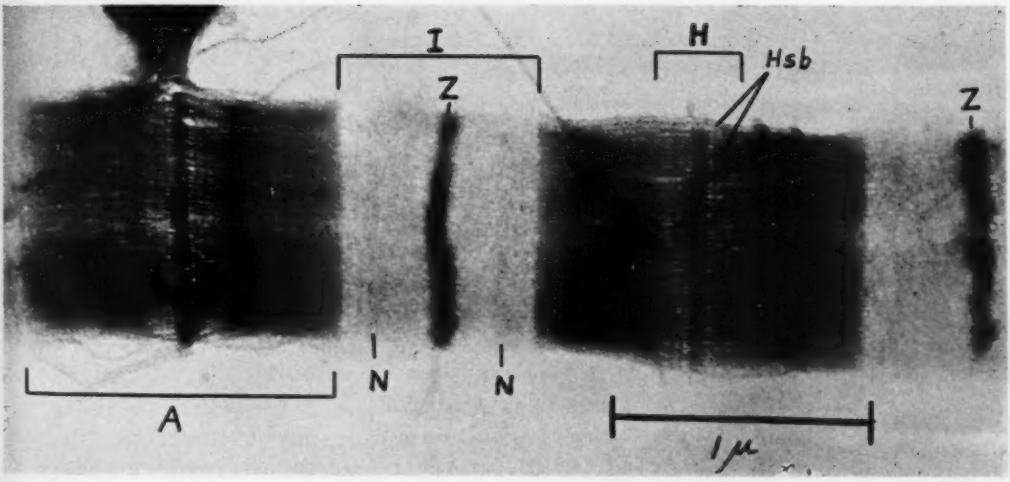

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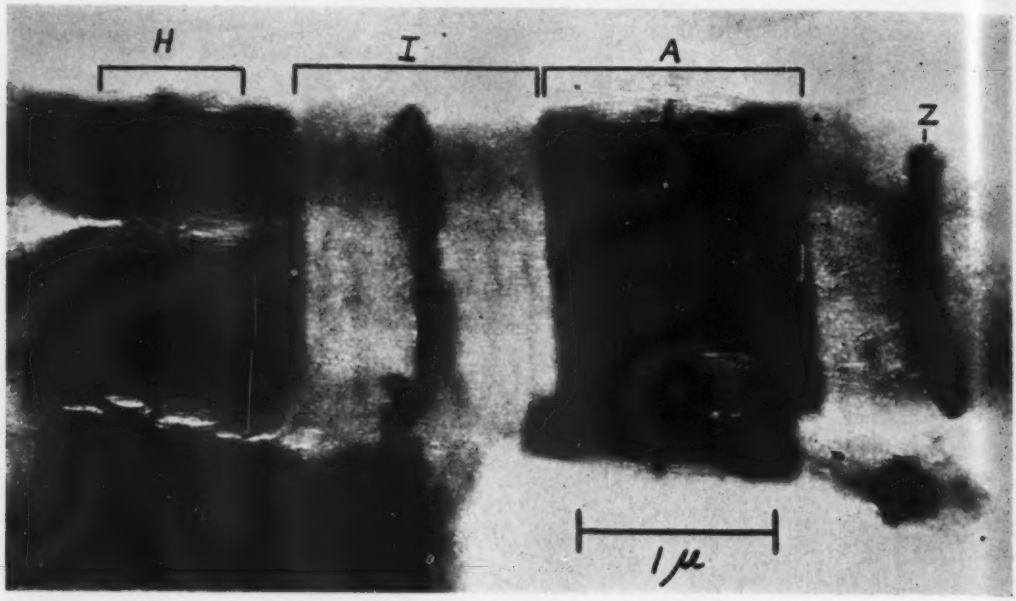

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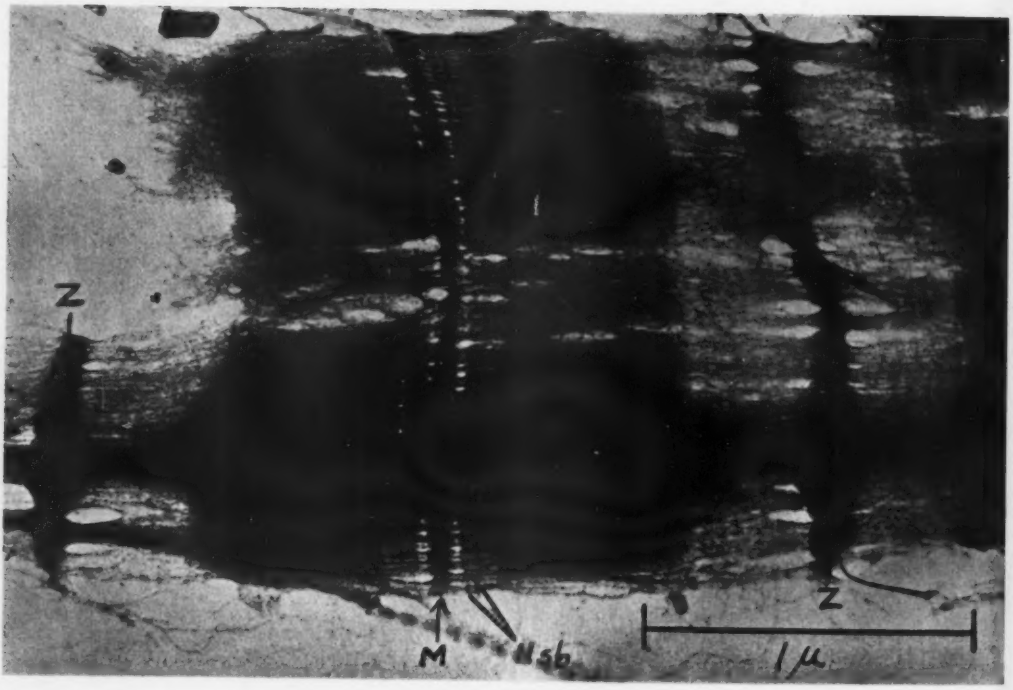

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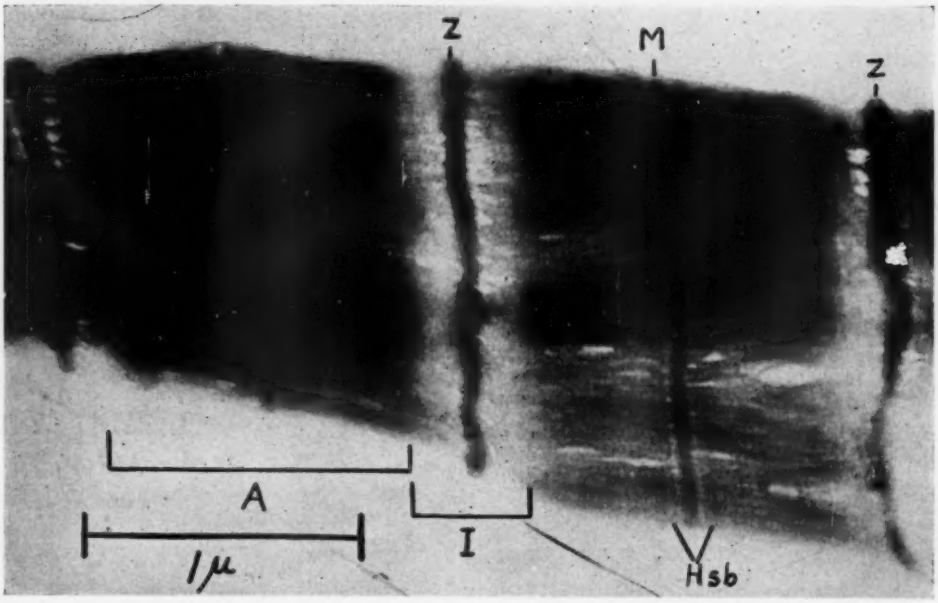

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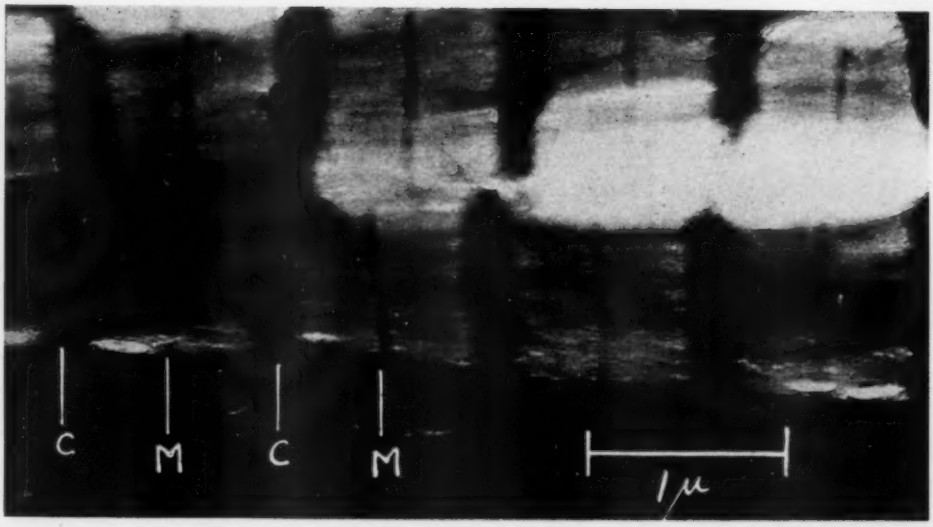

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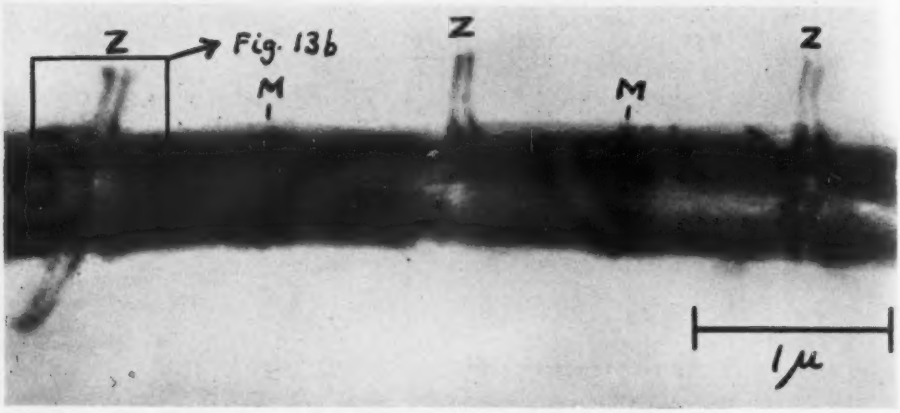

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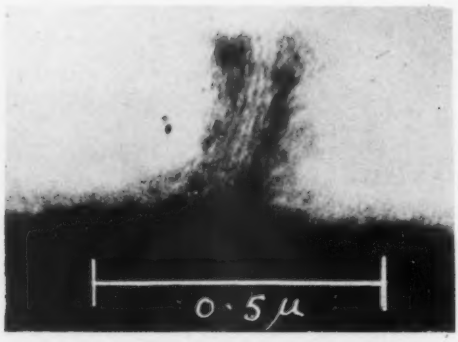

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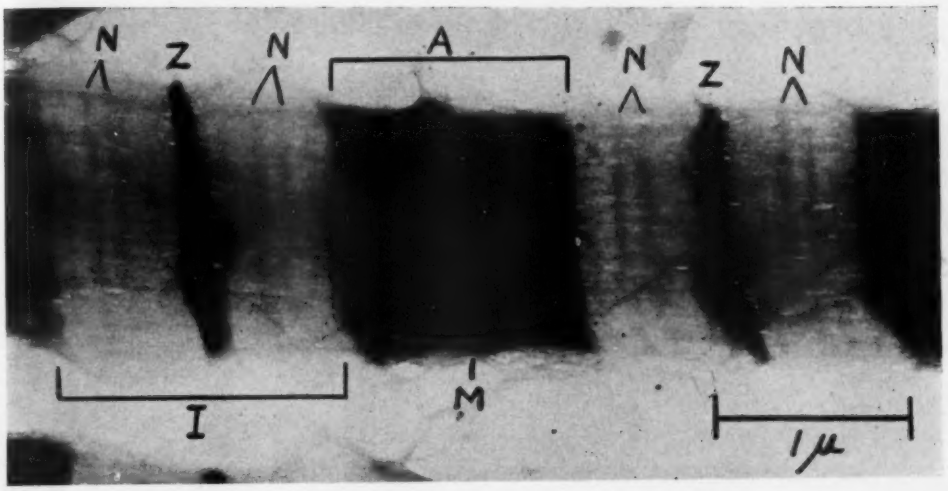

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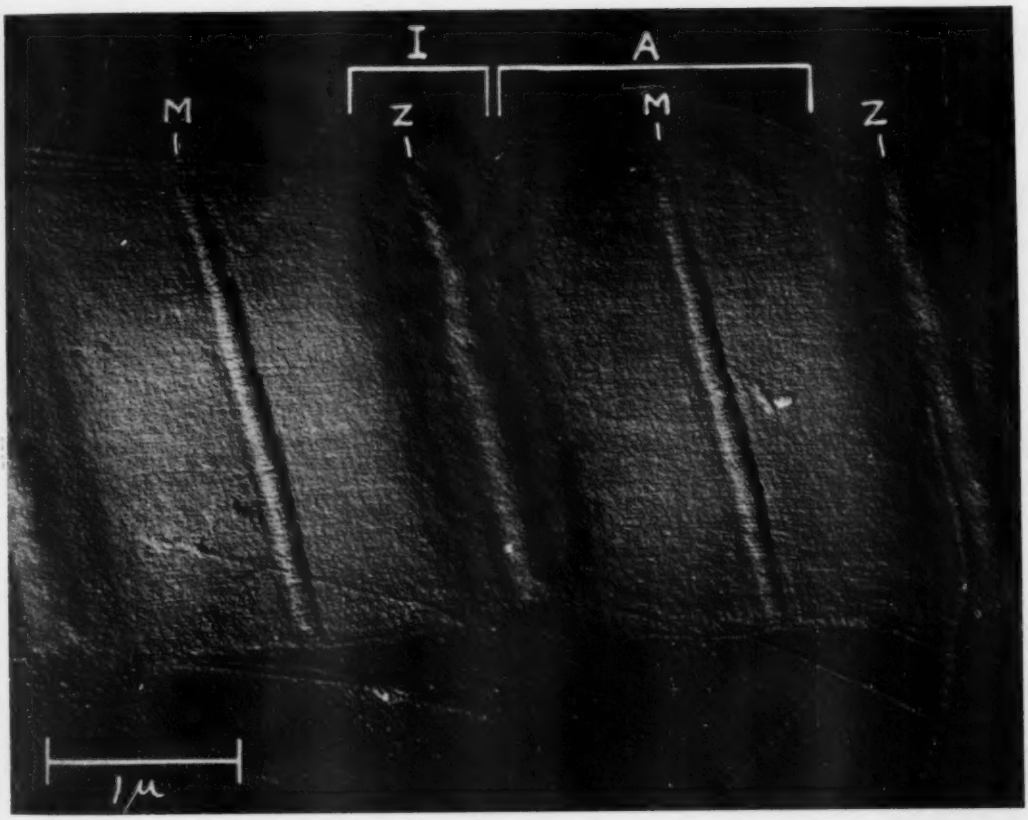

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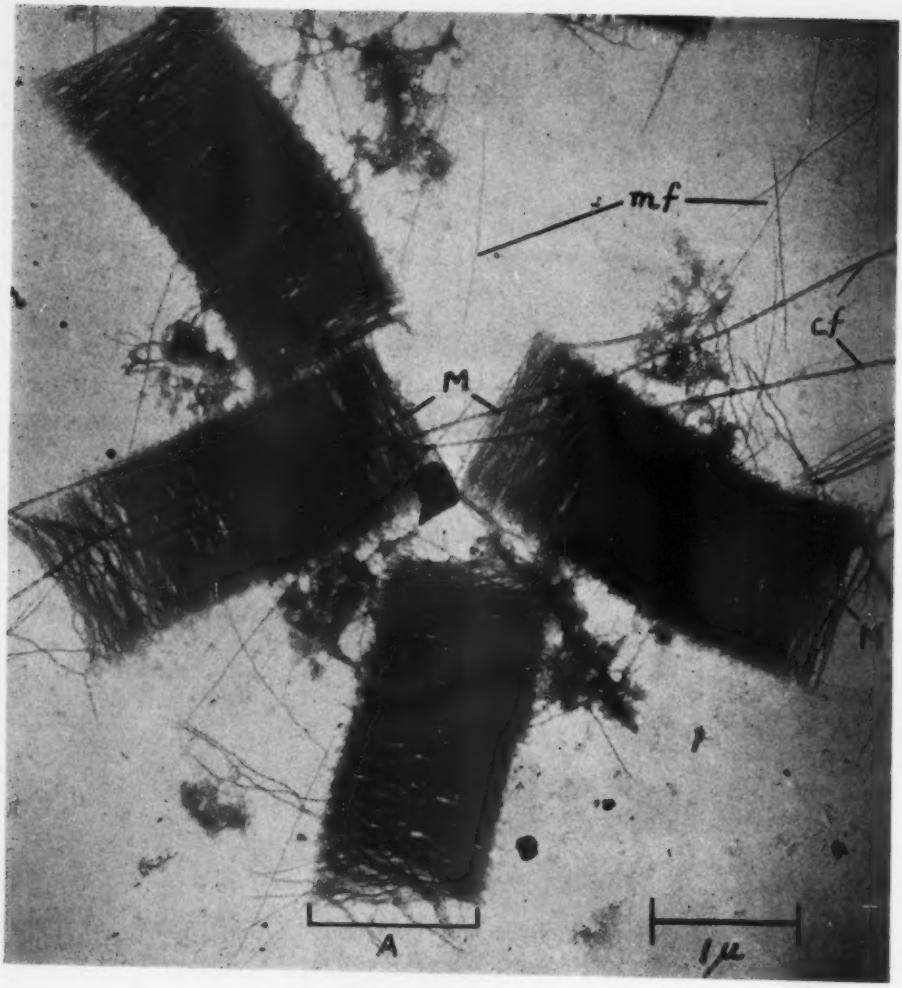

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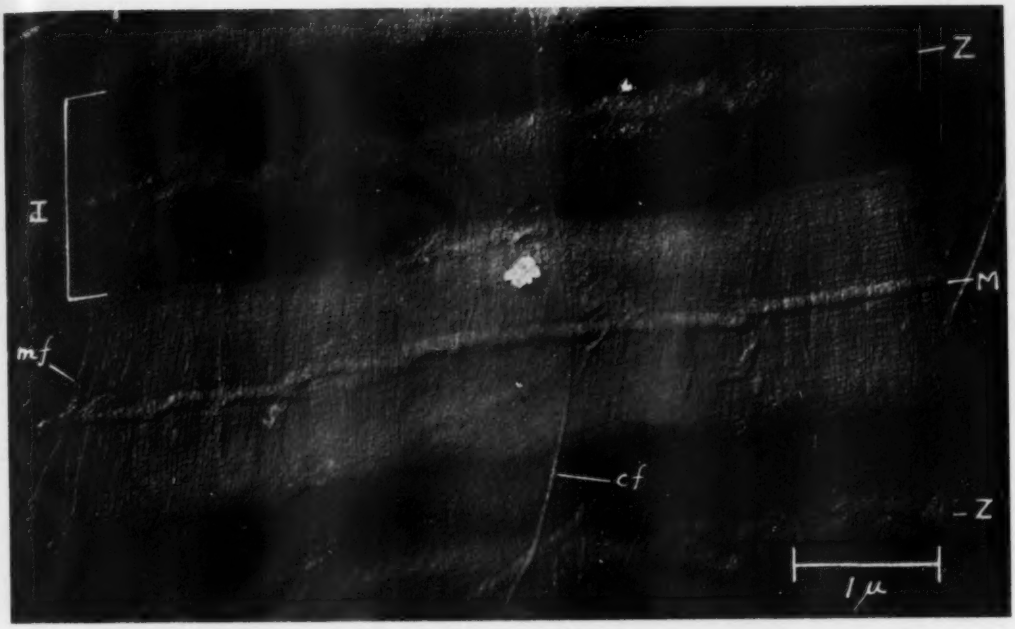

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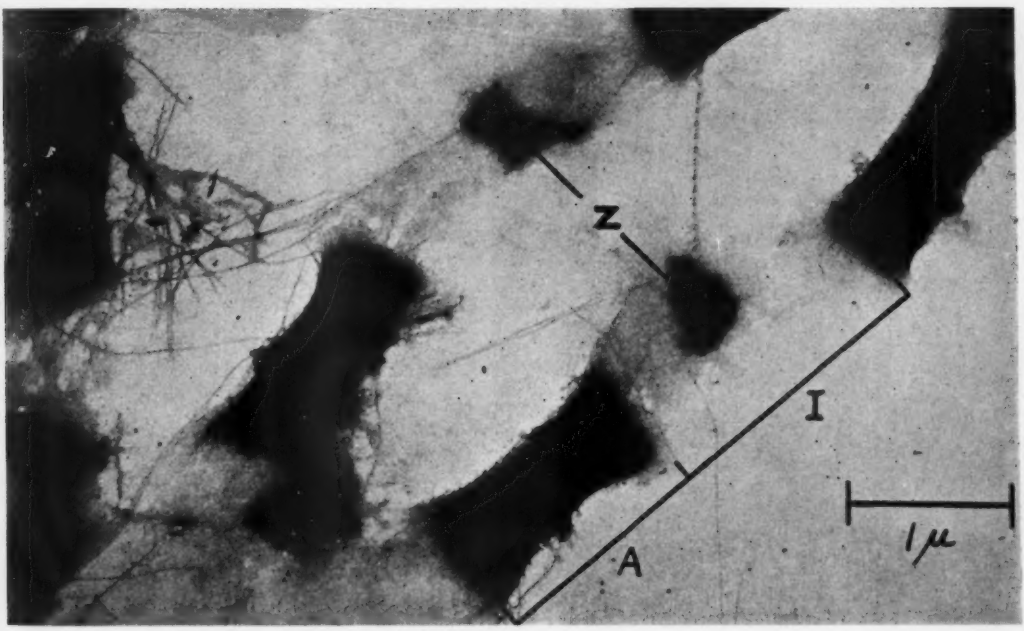

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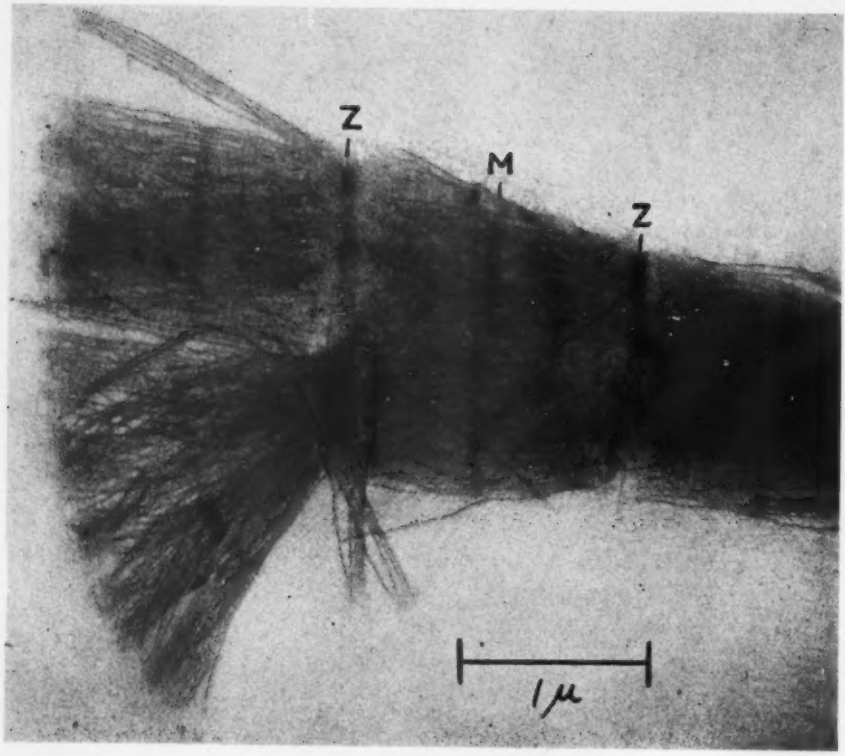

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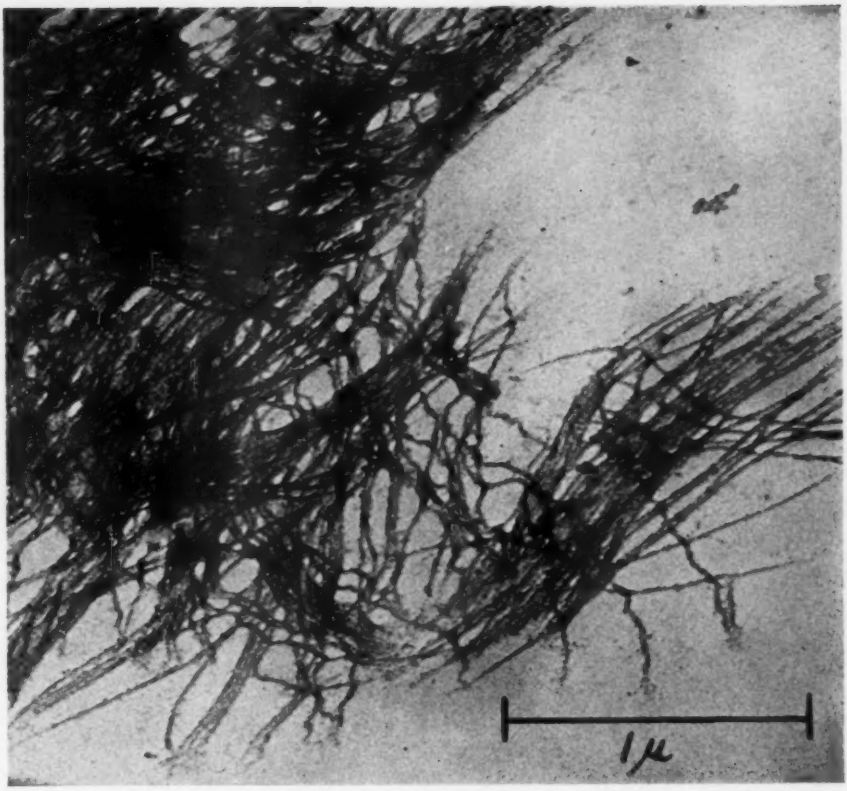

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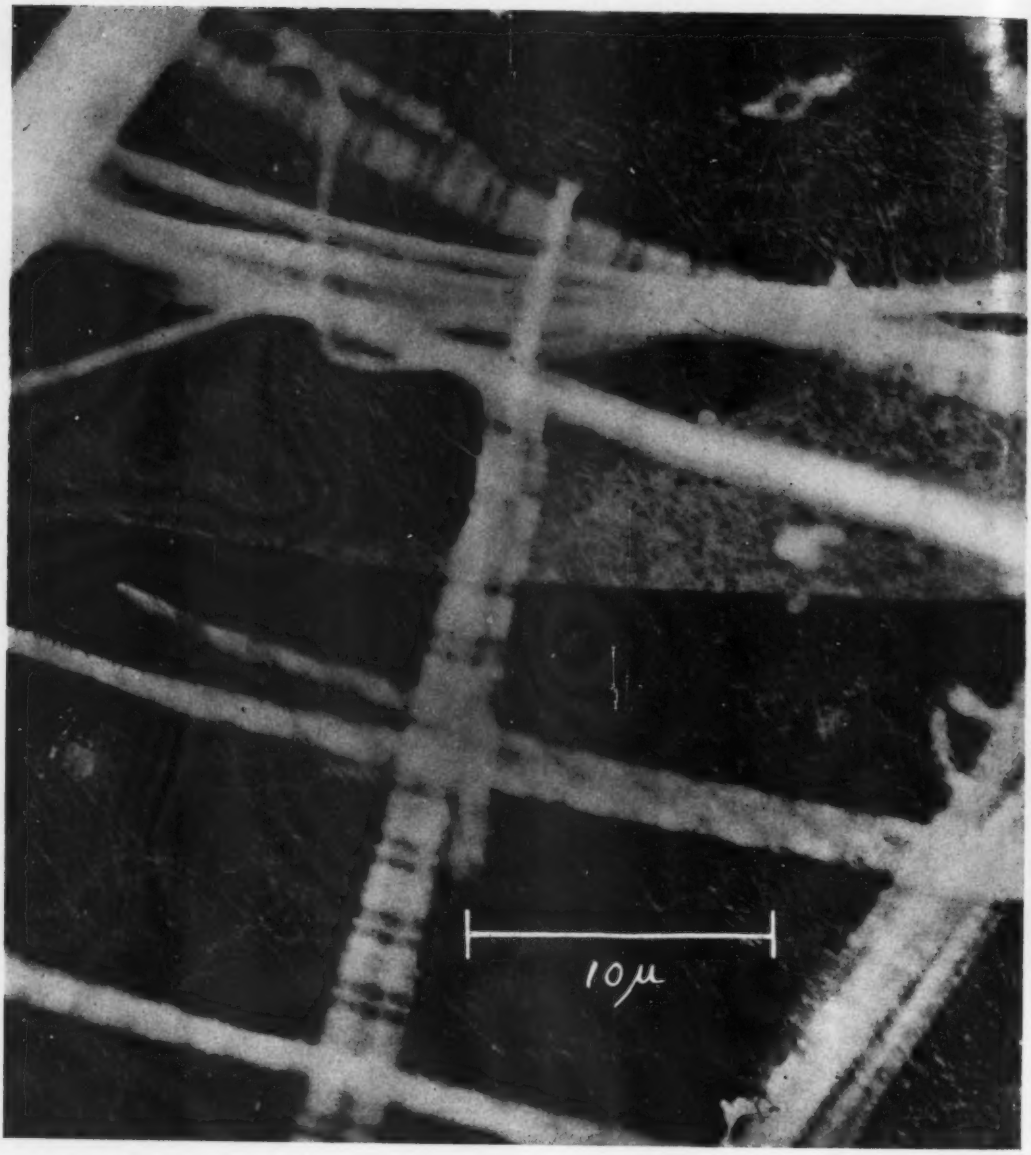

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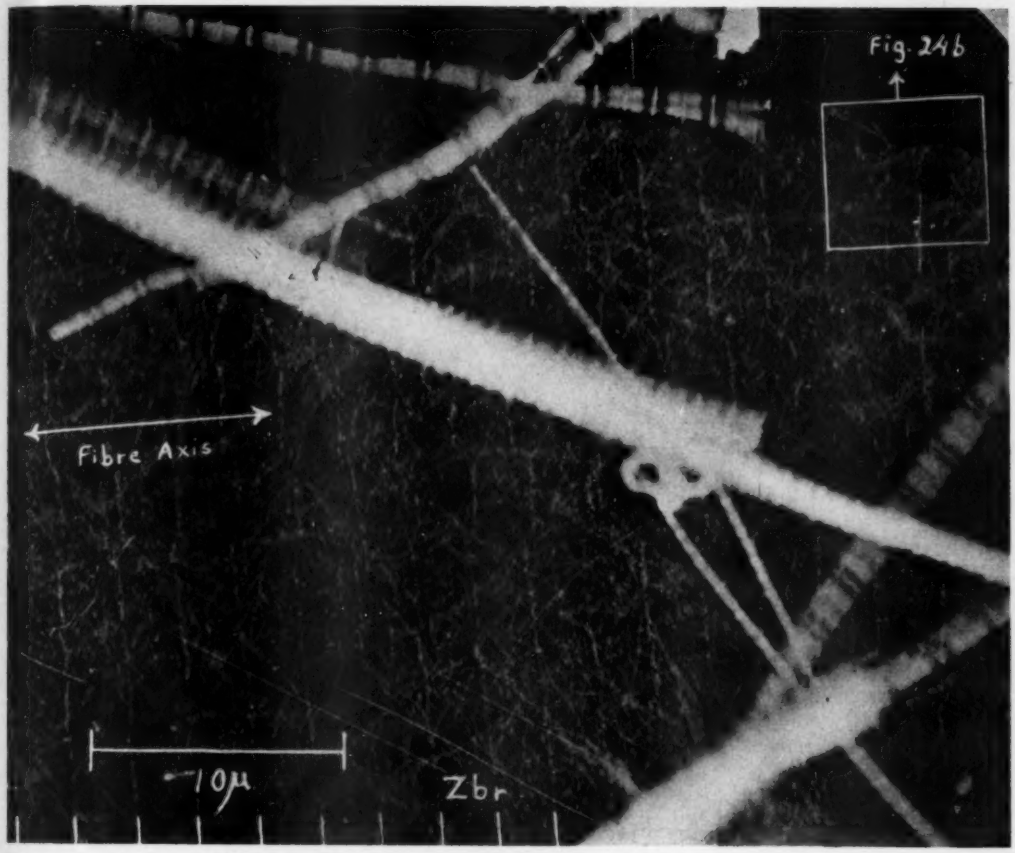

Fig. 22.

Fig. 23.

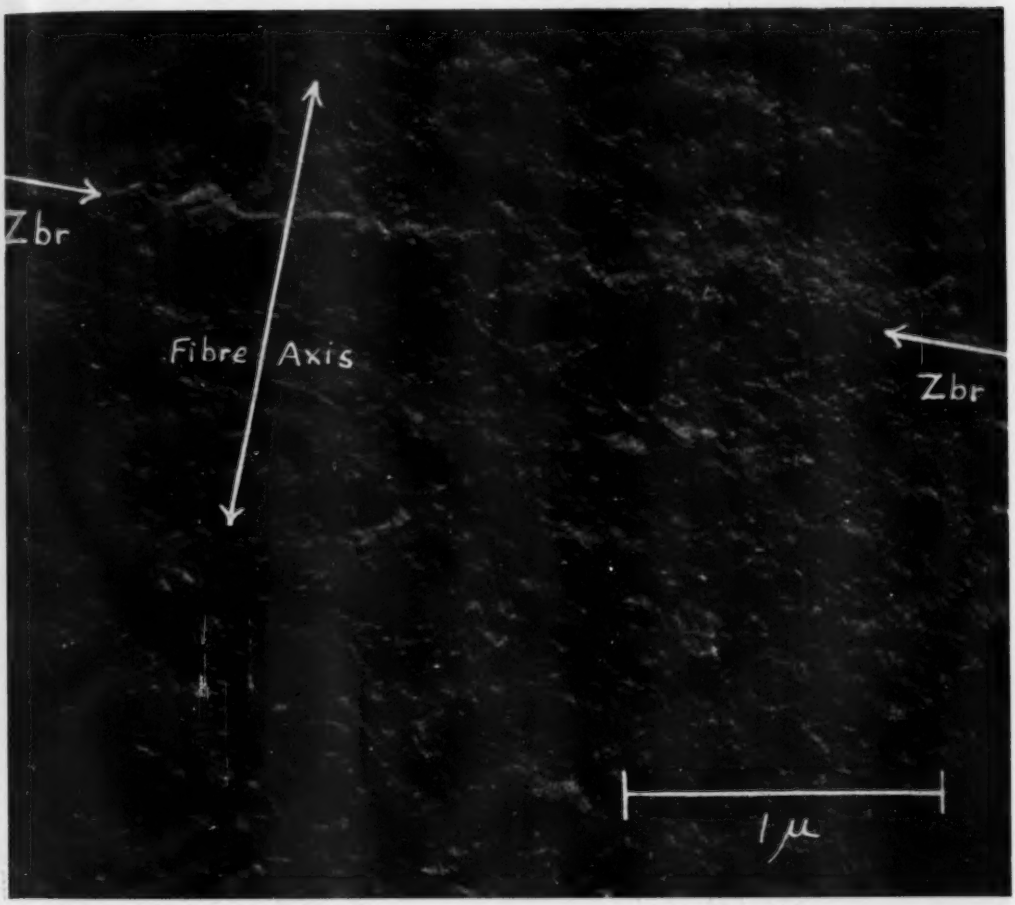

Fig. 24a.

Fig. 24b.

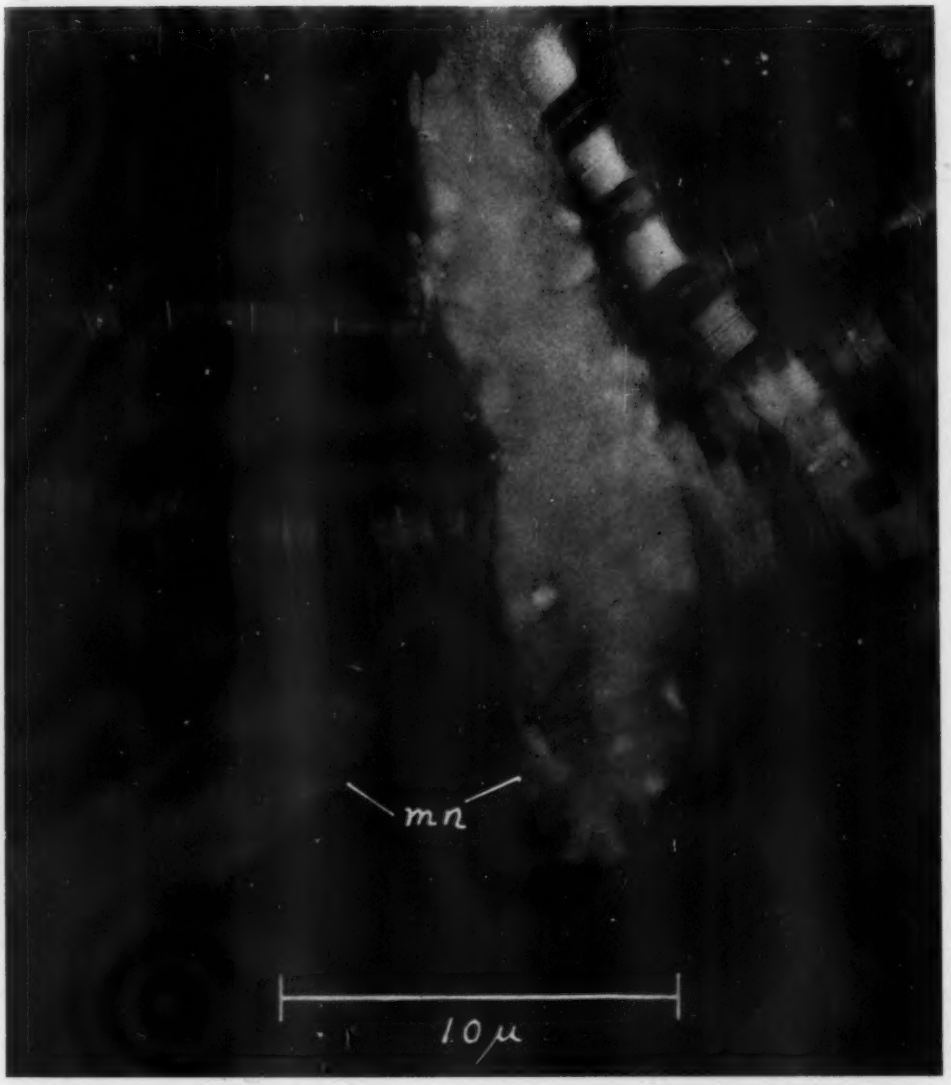

Fig. 25.

THE MECHANISM OF EASY STANDING BY MAN

by IAN W. KELTON AND R. DOUGLAS WRIGHT[1]

(From the Department of Physiology, University of Melbourne).

(Accepted for publication 27th May, 1949.)

During last century the theory of the standing posture of the human body formed a self-contained chapter of pure physiological statics. Because the body continually sways to a much greater extent than can be reconciled with the concept of a passive equilibrium, the body collapses when muscular action is stopped and standing is associated with increased metabolism, du Bois-Reymond (1905) came to the conclusion that standing is not a position of rest but depends on muscular action. With the growth of knowledge of the phenomena of decerebrate rigidity in cats and dogs, Sherrington stated in 1910 that the acts of standing and progression were of such importance to existence that probably ".... in essence the organization of these acts must, even in man, be purely reflex". Magnus (1926) in reporting his results and conclusions from a study of static reflexes in a variety of decerebrate laboratory animals states that in easy standing in man each part of the limb and the body as a whole is supported by tonic muscular contractions evoked reflexly; the part played by ligaments is dismissed as of little importance. Generally, this conclusion has been accepted for man and the stretch reflex discovered by Liddell and Sherrington (1924) has been accepted as the basic reflex for standing.

There have been observations which have thrown some doubt on the validity of these conclusions. Hoefer (1941) found practically no electrical activity in the anterior tibial and gastrocnemius muscles in seven human subjects in the easy standing position, and concluded that the elastic properties of muscles might be sufficient to maintain the standing position. Jacobsen (1943) criticized Hoefer's finding on the grounds that the apparatus which he used was not sufficiently sensitive to detect action potentials even if they were present. Abbie (1946) states that standing may be associated with relaxed muscles. Wright (1947) reported stable easy standing in a patient with muscular dystrophy of such degree that the patient had to be lifted and adjusted into the standing position by another person.

This problem was investigated with the following considerations in mind. From the experience of the case referred to above, a general consideration of the anatomical structures involved and the information recorded in the older

[1] This work was assisted by a Grant from the National Health and Medical Research Council.

papers, the conclusion that there is an easy standing position for man which is based upon osseous and ligamentous arrangements was adopted as the hypothesis under investigation. The validation of this hypothesis requires:

(a) the definition of the easy standing position;

(b) evidence that this position is maintained for considerable periods of time in the absence of muscular action;

(c) description of the osseous and ligamentous distribution which is mechanically adequate for such mechanical standing.

The Easy Standing Position.

The easy standing position is with the shod feet separated laterally by about the inter acetabular (pelvic) distance and the toes turned out by approximately 25°. The knees are locked and the hips fully extended; the lumbar and cervical vertebrae are convex forward and the thoracic vertebrae convex backward. The head may be dorsiflexed or ventriflexed at the atlanto-occipital joint. The arms hang easily by the sides. The exact angles at each joint are not described because they vary to some extent between individuals. Many people cannot adopt the easy standing position without instruction, but once they have found it they can repeat it. Children and adolescents adopt it readily, but it is not a fashionable position and ex-military personnel, especially officers, call it sloppy and are reluctant to adopt it. All muscles are relaxed. One point which is commonly overlooked is the outward pointing of the toes. The patient previously described by one of us (Wright, 1947) was not stable in the standing position unless the feet were in this position.

Evidence that the Easy Standing Position can be Maintained for Considerable Periods of Time without Muscular Activity.

The evidence obtained in this respect was of two sorts: (a) Palpation. (b) Electrical activity.

Palpation.

It is a simple thing to feel the tendons and muscle masses when the subject is in the easy standing position. The evidence so gained is, however, very cogent. A large number of subjects has been observed. In all of them the muscle masses were flaccid and the tendons loose. The groups of muscles examined were: anterior and posterior leg muscles, anterior and posterior thigh muscles, gluteal muscles, femoral adductor muscles, abdominal muscles, dorsal spinal muscles, neck muscles and mandibular muscles.

Electrical Activity.

Methods.

Three types of electrodes were used.

(i) Surface electrodes of stainless steel 2 cm. x 2 cm.

(ii) Coaxial electrodes of the type described by Adrian and Bronk (1929) with the modification that, for better insulation, the wire core was passed through finely drawn glass capillary tube within the needle shaft.

(iii) Hypodermic needles dipped in thin lacquer and baked for 2 hours at 100° C. This process was repeated and the area required for contact with the muscle tissue bared by removing the lacquer with a razor edge (Gilson and Mills, 1941; Dentlow and Hassett, 1943).

When surface electrodes were used one was placed on the skin overlying the muscle to be examined and the other placed, as an indifferent electrode, preferably over some non-muscular area near the muscle. Electrolyte paste was used to establish good contact with the skin.

Needle electrodes with up to 1 cm. of their shafts stripped of insulation were inserted in pairs into the muscle to be examined. The shafts of the needles were placed approximately 1 cm. apart.

For observations on single muscles either an Offner type 140 amplifier or a four-stage, resistance-capacity coupled, differential amplifier with a frequency response flat from approximately 0·25 to 5,000 c.p.s. was used. The maximum voltage gain available was greater than 1×10^6 with a noise level of less than 2 μV.

The amplifier output was connected to the deflector plates of a Cossor 339 oscillograph.

Two Offner, type 140, amplifiers were used for making observations on pairs of muscles simultaneously, their outputs deflecting the separate beams of a Cossor double-beam oscillograph.

The subject, wearing normal shoes, adopted a relaxed standing position with the feet comfortably spaced well apart, toes turned out slightly and abdominal muscles relaxed.

Needle electrodes were used in all muscles examined and in addition, surface electrodes were used on many of the superficial muscles.

For a varying period after the insertion of the needle electrodes bursts of "insertion motor unit potentials" as described by Weddell *et al.* (1944) were frequently observed.

To test the evidence from electromyography in regard to sensitivity, electrodes were implanted in the belly of the m. flexor digitorum profundus of the right middle finger as tested by using the electrodes for stimulation. Then with the distal phalanx of the finger at 40° inclination to the hand, wrist and proximal phalanges, which were supported horizontally, weights were placed on the finger tip. Up to 40 gm. load no electrical activity occurred, but on increasing the weight further occasional action potentials appeared. The maximal weight which the finger could support in this position was 8 kg., so that action potentials appeared at 1/200th of this maximum. There is no information as to the number of nerve fibres going to these groups of muscle fibres so it is not possible to say whether this corresponds to one motor unit, but on nerve size and fibre counts in comparable nerves this corresponds to not more than 10 motor units. Allowing for leverage, the 40 gm. is within the motor unit dimensions of Eccles and Sherrington (1930).

This experiment was repeated with two electrodes in that part of the m. flexor digitorum profundus which flexes the distal interphalangeal joint of the fifth digit.

It was found that the maximal weight which could be supported by the flexed terminal phalanx was 2,500 gm.

The maximal weight supported without the appearance of m.u.p.'s was 10 gm.

Electromyography is therefore a very sensitive indicator of activity in the muscle.

Results.

The following muscles were examined in two subjects:—

Lateral crural m.	peroneus longus
Posterior crural mm.	gastrocnemius soleus flexor hallucis longus
Anterior crural mm.	extensor digitorum longus tibialis anterior
Posterior femoral mm.	biceps femoris semitendinosus
Medial femoral mm.	gracilis adductor magnus
Thigh mm.	tensor fasciae latae sartorius rectus femoris vastus medialis

In addition, the following muscles were examined in one of the subjects.

Foot mm.	abductor hallucis flexor digitorum brevis
Lateral crural m.	peroneus brevis
Posterior crural mm.	flexor digitorum longus tibialis posterior
Anterior crural m.	extensor hallucis longus
Posterior femoral m.	semimembranosus
Gluteal mm.	gluteus maximus gluteus medius
Medial femoral m.	adductor longus
Thigh m.	vastus lateralis
Trunk mm.	sacrospinalis latissimus dorsi trapezius (thoracic part) rectus abdominis external oblique abd.
Neck mm.	splenius group semispinalis group trapezius (cervical part) scalenus levator scapulae sternocleidomastoid
Mandibular mm.	temporal (anterior fibres) masseter

All of these muscles except the tibialis anterior and the soleus were electrically silent for long periods of time.

The soleus and anterior tibial mm. were simultaneously inactive for periods usually of 1 to 5 seconds duration, with infrequent periods of up to 32 seconds.

The soleus m. was inactive for periods of 1 to 5 seconds with rare periods of longer duration.

The anterior tibial m. was inactive for periods varying from 1 second to 3 minutes, the period usually being greater than 30 seconds.

Periods of activity in the soleus m. ranged from 5 seconds to 3 minutes and in the anterior tibial m. from 2 to 40 seconds.

Description of the Osseous and Ligamentous Distribution which is Mechanically Adequate for Mechanical Standing.

From the older literature (du Bois-Reymond, 1905) the centres of gravity of the parts above the thighs in the easy standing position are:

(1) *Head.* 5 mm. in front and 4 mm. above centre of rotation of atlanto-occipital joint and 67·5 cm. above and 1 cm. dorsal to the axis of rotation of the hip joints.

(2) *Trunk.* 24·8 cm. above and 0·6 cm. dorsal to the axis of rotation of the hip joints.

(3) *Trunk, head and arms combined.* 18 cm. above and 0·86 cm. dorsal to the axis of rotation of the hip joints.

With the body in the easy standing position the centre of gravity of each relevant portion of the body projects through the junctions of the curves of the vertebral column. The following factors render the cervical and lumbar vertebrae stable but elastic when convex ventrally and the thoracic vertebrae stable but elastic when convex dorsally.

(1) The shape of the articular facets.
(2) The shape of the bodies of the vertebrae and the intervertebral discs.
(3) The anterior longitudinal ligaments.
(4) The inter-laminar ligaments.
(5) The shape of the spines and distribution of the interspinous ligaments.

In order to get some idea of the dimensions of the elasticity of the vertebral column lateral roentgenograms of a subject in the easy standing position were made. The hands were then loaded with 20 kg. each and the easy standing position regained. Further roentgenograms showed that the thoraco-lumbar curve (T_9–L_3) had bowed forward a distance of 3 mm. over a length of 21 cm.

There is no need to refer to the anatomy of the sacral attachments to the os coxae. The mechanical stability of this region is unquestioned.

The hip joint in the easy standing position is stressed by the weight of the upper part of the body causing extension of the joint. This is limited by the vertical portion of the iliofemoral ligament. This statement was tested in the autopsy room. On one side the limit of extension of the hip was noted and the fascia femoris, iliopsoas, rectus femoris and adductor muscles divided. Extension was not significantly affected. The iliofemoral ligament was then divided and the thigh could be folded back on the trunk. On the other side the ligament and muscles were divided leaving the fascia femoris intact. Full hyperextension was possible, but adduction was limited by the fascia when the hip joint and knee joint were extended. This finding is probably associated with the great lateral stability of the standing position (see later). If we consider a body set up and hinged to two legs as in Fig. 1a, it can readily be

seen that any lateral deviation must result in collapse. In order to stabilise it ties can be fixed inside or outside the legs. When fixed outside the legs they must be so disposed that movement at the hinge must strain the tie. The disposition of the fascia femoris in relation to the iliac crest and the trochanter major gives this result when the hip is extended. (Fig. 1b).

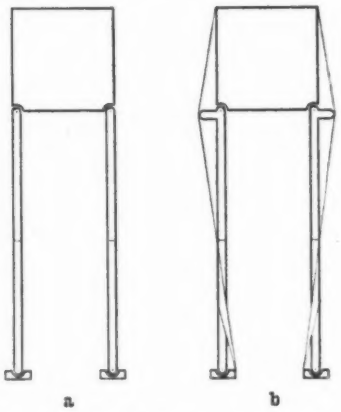

Fig. 1. Block diagram to show the effect of the trochanter and antero-medial insertion of m. tibialis anticus. Sway to one side with the trunk vertical stresses the ipsilateral fascia femoris and m. tibialis anticus.

At the knee joint the centre of gravity of the combined thigh and trunk falls in front of the centre of rotation of the condyles. Fick (1911) gives gravimetric evidence for this and it can be tested by the old schoolboy trick of surreptitiously moving the knees of the relaxed subject forward. A forward movement of the range of 1·5 cm. causes the mechanism to collapse. The basic mechanism here is that each femoral condyle has a dorsal part circular about a horizontal transverse axis and the collateral ligaments of the knee are attached to the centres of these circular parts. The medial condyle has also an anterior portion curved about a vertical axis centred on the distal part of the saggittal curve of the lateral condyle. When the knee is fully extended it is then locked by rotation about this vertical axis bringing the femoral attachment of the medial collateral ligament further posteriorly relative to the tibia. Because of the forward projection of the lateral condyle hyper-extension cannot now take place because the collateral ligaments of the knee joint prevent the dorsal points of the femoral condyle from lifting from those of the tibia. This was tested in the autopsy room. Section of the collateral ligaments allows hyper-extension: section of all other ligaments leaving the collaterals intact does not permit hyper-extension.

It should be noted at this point that when locking takes place the tibia is fixed to the foot which is not moved on the floor. The femur is therefore rotated relative to the tibia and the body. This internal rotation of the femur decreases the extension possible at the hip joint as may be tested on the living subject. Locking the knee may therefore be said to increase the stability of the hip joint.

The mechanism giving stability at the ankle is not so simple nor indeed so certain as at other joints. The factors which appear to operate are as follows:

(1) The shape of the saggittal curve on the superior surface of the talus is not circular but of a parabolic form. If the body is to move forward the body must be lifted vertically, and in easy standing the centre of gravity of the body falls just in front of the centre of rotation of the ankle joint, i.e. the tarsal attachments of the collateral ligaments.

(2) The superior surface of the talus is wedge shaped with the wide end forward. It is an obvious possibility that as the foot rolls inward in a relaxed condition under the weight of the body, the movement of the bones on each other alters ligamentous stresses and locks the tibiofibular gate against further dorsiflexion of the ankle. We have been unable to obtain evidence of this. Amputated legs were mounted in an apparatus with a footrest having the same rotation as the ankle. Weights up to 40

kg. were applied to the top of the tibia. Increasing weight did not increase the force required to shift the foot from its stable position. The factors mentioned above under (1) and (2) were, however, clearly shown.

(3) A factor of some importance is apparently the placing of two ginglymus joints with their axis at 45° and their arms firmly fixed, one of each to the earth and one of each through the legs, thighs and pelvis to each other. This was important in the case previously reported but is not a rigid mechanism. As much as 20° dorsiflexion of the ankle can take place while these conditions are maintained.

No claim is made that these mechanisms at the ankle provide stability over a wide range. It was decided to test what range at the ankle could be accommodated without muscular activity being called upon to restore mechanical equilibrium. First it was necessary to find which muscles first become active when the body sways antero-posteriorly.

Several muscles were connected in pairs through Offner amplifiers to a twin beam Cossor C.R.O. with the subject out of view of the screen. As each sway developed the muscle first active was noted. The leg muscles were first in action with tibialis anticus and soleus well ahead of gastrocnemius which commonly did not become active before equilibrium was re-established. Having this information the following method was adopted.

To determine the angle through which the subject swayed on the ankle joints without motor unit potentials being evoked from the muscles a combined attack was used: electromyographic and gravimetric. The platform was removed from a 0–200 lb. weighing machine of the "jockey scale" pattern and a standing board clamped on to the levers in such a position that the sensitivity of the balance system increased as a weight was moved towards the steelyard arm.

A transverse line was drawn across the centre of the board and a 20 kg. weight placed on the board with its centre of gravity exactly over the line. The reading on the scale was noted. The centre of gravity of the weight was then moved to a position 1 cm. forward of the line and then to a position 1 cm. behind the line and scale readings noted for each position.

The readings were: $\underset{92{\cdot}5}{-1\text{ cm.}}$ $\underset{96{\cdot}5}{0}$ $\underset{100{\cdot}5}{+1\text{ cm.}}$, so that a variation of 1 cm. gave a deflection of 4 units on the scale.

The body weight of the subject was 64·5 kg. and therefore a variation of 1 cm. in the position of the subject's centre of gravity would give a deflection of $\frac{64{\cdot}5}{20} \times 4 = 12{\cdot}9$ units on the scale.

The subject stood on the board with the line 5 cm. in front of the ankle joint, which is considered by some to be the position of a vertical line through the centre of gravity of the body. (The exact position of the centre of gravity is of no importance in this experiment.)

A pair of needle electrodes was inserted into the soleus m. and a pair into the anterior tibial m. and the leads from them connected to two amplifiers the outputs of which deflected separate beams of a double beam oscilloscope.

The soleus and anterior tibial muscles were chosen as they were seen in previous experiments to be the first muscles to become active in correcting instability due to postural sway.

Some time was allowed for the "insertion motor unit potentials" to die away and for the subject to become accustomed to a relaxed standing position on the board.

Readings in pairs were taken from the scale on the initiation of activity in each muscle for single cycles of postural sway.

A series of readings were taken and the differences between readings in each pair were averaged.

Scale readings when m.u.p.'s appeared in muscle.

Anterior tibial	Soleus	Difference
78	86	8
76	82	6
72	80	8
77	85	8
76	84	8
80	89	9
78	87	9
80	87	7
78	87	9
70	79	9
73	82	9
71	79	8
73	79	6
70	79	9
75	82	7
72	79	7
80	87	7
78	87	9
74	82	8
76	83	7
72	81	9
	Total:	167

Average difference between readings: 7·95.

As a difference of 1 cm. in the position of the centre of gravity of the subject was estimated to give a difference of 12·9 units in the scale reading, the centre of gravity of the subject was calculated to have moved $\frac{7{\cdot}95}{12{\cdot}9}$ cm. = 6·1 mm. between the positions at which the soleus and anterior tibial muscles became active.

Taking the level of the centre of gravity to be about the middle of the body of the last lumbar vertebra (Robinson, 1931) this was found in the subject to be 89 cm. above the axis of the ankle joint. From these measurements, the angle through which the subject swayed on the ankle joints without muscular activity present was calculated to be 0° 24′.

Using the same apparatus and with the subject facing at 90° to the previous stance, side to side sway was measured. Three components were observed in side to side sway:

(a) An extremely small sway which represented a movement of less than 0·5 mm. at the level of the subject's centre of gravity. This was equal to a rotation about the ankle joint in a side to side plane of less than 0° 2′. The frequency of this sway was 2 to 3 per second.

(b) A fairly regular sway representing a movement of 1·6 mm. at the level of the centre of gravity or an angle of 0° 6·2′ at the ankle joint. The frequency of this sway approximated very closely to 1 per second.

These minor movements described in (a) and (b) were so regular that they appeared to be recoil effects from cardiac action.

(c) A very irregular greater sway involving an average movement of 5·1 mm. at the level of the centre of gravity or an angle of 0° 19·8′ at the ankle joint. The frequency of this type of sway was most irregular, averaging approximately 3 per minute and reflected antero-posterior sway.

As reported earlier in this paper, the adductor and abductor muscles of the thighs were electrically silent during easy standing and it therefore appears that the mm. tibiales anteriores are effective not only in stabilising antero-posterior displacement, but also lateral sway. Reference to Fig. 1 will reveal that the attachment of this muscle medial to the centre of rotation of the talo-calconeal joint allows this action. The line of action of this muscle is in the line of the fascia femoris.

Stretch Reflex.

It is of some interest in this connection to compare the ease of eliciting the stretch reflex from such muscles as the tibialis anticus and the soleus with that of the quadriceps femoris which is not called on to correct postural sway. Needle electrodes were placed in these three muscles and the ankle or knee flexed or extended by a manipulator while a third person observed the results.

For observation of the stretch reflex in the soleus and tibialis anticus the foot was firmly fixed to a short board which was hinged at the axis of the ankle joint by means of metal uprights. As the board was rotated by hand around the hinge it operated a vertical writing point on a kymograph drum. The kymogram was calibrated in degrees of rotation before each experiment and a timing trace also recorded. The muscle was loaded by exerting a pull in the required direction by means of weights attached to the toe of the board which was steadily resisted by the subject, and the reflex tested after removal of the weights.

When observing the quadriceps femoris the leg was supported in a horizontal position almost to the knee. The lower leg was supported passively and allowed to drop through varying angles.

Results.

Soleus m. The minimum movement found necessary to produce reflex activity was 18° rotation in 0·2 sec.; 12° rotation in 0·125 sec.

After the muscle had been loaded for 3 minutes with 10 kg., reflex activity was produced by 5° rotation in 1·0 sec., 7° rotation in 3·5 sec., 2° rotation in 0·75 sec.

Tibialis anticus m. No reflex activity was observed when the foot was plantar flexed through an angle of 45° in 0·2 sec. After loading the muscle for 3 minutes with 5 kg., reflex activity was observed when the foot was extended through 34° in 0·2 sec.

Quadriceps femoris m. The leg had to fall through an angle of 90° at the knee joint before any reflex activity was observed.

Loading the muscle with 5 kg. at the level of the ankle for 2 minutes with the extremity horizontal and supine made no apparent difference in reflex activity.

Other results. Loading with 20 kg. for ten minutes lowered the threshold for the Knee-Jerk or increased the response to a given stimulus. This facilitation was limited to the muscle which had been loaded and gradually decreased to basal over a period of twenty minutes.

DISCUSSION.

It would appear that a notion which started with an erroneous interpretation by du Bois-Reymond (1905) of the mechanism of the knee was then furthered by interpretations transferred from decerebrate animals to intact man. None of the laboratory animals has an easy standing position. Their

standing position is part of a stalking or rage reaction. Indeed, decerebrate rigidity with its high blood pressure, high blood sugar and bodily posture could be interpreted more consistently as a mutilated rage reaction than as reflex standing if one wished to make such suggestions. Adult man has an easy standing position and does not immediately fall from it on going to sleep. Only a few animals have this characteristic, notably the adult horse and a number of birds.

The evidence here adduced follows the argument previously outlined. There is no evidence to the contrary. There are only assertions derived from decerebrated animals which have different habits and correspondingly different skeletal and ligamentous arrangements.

It would appear therefore, that easy standing in man depends primarily upon mechanical arrangements, but at the ankle these are not highly developed and muscular activity is reflexly excited when the joint departs from the mechanical equilibrium position by a small angle. This, however, cannot be a simple stretch reflex—it occurs with an angular motion much less than was found necessary to elicit the stretch reflex even after facilitating the reflex by preliminary loading.

The apparatus used for electromyographic recording did not possess characteristics which would detect the small junction potentials reported by Gerard and Kuffler. Such activity is not proved to have any effect in mammalian muscle and the absence of tension in the tendons during standing also indicates the lack of effective muscular action.

SUMMARY.

In the easy standing position the only muscular activity is in the tibialis anterior and the soleus muscles. Here the activity is intermittent and associated with restoration of the mechanically stable position. Activity commences in these muscles when they are deformed to a much less extent than is required to elicit a simple stretch reflex.

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THE THERMAL DESTRUCTION OF VITAMIN B_1

6. THE EFFECT OF TEMPERATURE AND OXYGEN ON THE RATE OF DESTRUCTION OF ANEURIN

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The only attempt thus far to determine the effect of temperature on the rate of destruction of aneurin is that of Rice and Beuk (1945), who studied the rate of destruction of aneurin in pork at temperatures from 121° C. to 49° C. They show a deviation from the Arrhenius equation (which relates log k and $1/T$) below 77° C. which could be related to enzyme activity. It has been shown in the earlier papers of this series that the effect of various factors on the rate of destruction of aneurin can be modified by the electrolyte system, i.e. by the various buffer solutions used to establish the pH, and, consequently, the effect of temperature on the reaction velocity of aneurin destruction has been studied in three different buffers. This work thus forms a continuation of the earlier investigations but opportunity was taken to determine whether there was any deviation from the Arrhenius equation below 77° C. in the absence of biological materials.

The simplest method of studying buffer solutions at temperatures greater than 100° C. was thought to be the use of sealed ampoules in which the solution under investigation could be heated in an oil bath. When this procedure was adopted with the phosphate buffer, which was studied first, the anomalous results which were obtained led to the discovery that oxygen was capable of accelerating the rate of destruction of aneurin, though to different extents in different buffer solutions. Thus, in yet another connection the importance of the electrolyte system is apparent.

EXPERIMENTAL.

Reagents.

The preparation of reagents was carried out in a similar manner to that used in earlier papers of this series (Farrer, 1947 a, 1947 b).

Buffer salts. The following solutions were made from A.R. reagents and distilled water and purified by the $CaCO_3$ absorption method of Stout and Arnon (1939): 0·5 M Na_2HPO_4; potassium hydrogen phthalate, KH_2PO_4 (0·5 M with respect to each salt). After filtration, the CO_2 was removed by boiling following the acidification of the solution with 6 N HCl (q.v.) Re-adjustment of the volume was carried out with twice distilled water after the solution had cooled.

Citric acid. A.R. citric acid was recrystallized three times from twice distilled water. A negative test was obtained for heavy metals using the dithizone method and an 0·5 M solution was therefore used with confidence.

6 N Hydrochloric acid. HCl (A.R.) was twice distilled from all glass apparatus.

Twice distilled water. Water was twice distilled from all glass apparatus with a "strip action" still.

Oxygen and nitrogen. Commercial gases were used without further purification except where stated.

Aneurin stock solution. Pure aneurin hydrochloride (10 mg.) was dissolved in acidified (HCl) twice distilled water and made up to 250 ml.

Method.

The Jansen thiochrome method was used. The procedure was that already described (Farrer, 1945) with suitable dilutions of the test solution to permit the reading of the fluorescence on the Coleman Model 12A Photofluorometer.

Procedure.

The appropriate buffer solution was prepared in a 200 ml. volumetric flask. 20 ml. of aneurin stock solution were added and the volume made up to 200 ml. with twice distilled water. The buffer solutions used were 0·1 M solutions of phosphate at pH 6·5, solutions 0·1 M with respect to both KH_2PO_4 and potassium hydrogen phthalate at pH 6·0 and McIlvaine phosphate-citric acid buffer solutions 0·05 times the concentration given by Britten (1932) at pH 6·3. The pH in each case was carefully adjusted from the slightly acidified buffer salt solutions with 6 N HCl using the glass electrode. It was rechecked at the conclusion of the experiment.

Where the solutions were to be heated under reflux they were transferred to a flask fitted to a reflux condenser by means of a ground glass joint and heated in an oil bath thermostatically controlled at the desired temperature to within ± 1° C. Samples were pipetted out at the required time intervals, cooled, diluted and assayed.

Where 10 ml. ampoules were used, five 10 ml. samples of the prepared solution were taken. In appropriate cases oxygen or nitrogen was bubbled through the samples for some minutes to displace all air from the solutions and "headspace" and the ampoules quickly sealed. Other wise the latter were simply sealed off retaining the air in the "headspace." All ampoules were placed in the oil bath together and allowed 5 or 6 minutes to come up to temperature. One was then removed ($t = 0$) and, at appropriate time intervals, the remainder were successively removed, opened and 5 ml. diluted to 100 ml. These diluted solutions were assayed.

RESULTS.

The method of interpretation is the same as that used in the earlier work (Farrer, 1945) calculating k, the velocity co-efficient, for aneurin destruction, from the first order reaction law, t being measured in minutes. The effect of temperature is shown by the Arrhenius equation:

$$\ln k = I - E/RT$$

where I, E and R are all constants, k is the velocity coefficient and T is the temperature in degrees Absolute. Graphically, conformity with the equation is shown by the linearity of the curve $\log k/{}^1/_T$. Such graphs have been constructed for the three buffers studied and are shown in Figs. 1, 2 and 3.

When this work was commenced, the first experiments were carried out at 110° C., 105° C. and 90° C. in an oil bath, using phosphate buffer solutions sealed in glass ampoules. The results of k obtained were used to plot the curve for the Arrhenius equation and it was at once seen that the value of k obtained earlier (Farrer, 1945) for the same buffer solution at 100° C. was well below the curve obtained. A fresh buffer solution was prepared and half was boiled and half filled into ampoules and heated in the oil bath at 100° C. The value of k obtained with the former confirmed the earlier result and the value obtained with the latter closely fitted the Arrhenius curve. Refluxed solutions (i.e. open to atmospheric pressure) were then placed side by side with the ampoules in the oil bath at the temperatures lower than 100° C. and the converging linear curves shown (Fig. 1) were obtained. The same value of k was obtained by each method at 70° C. and then at 60° C. and 50° C. The only explanation for this seemed to be that the oxygen sealed in the "headspace" or the ampoules as residual air was accelerating aneurin destruction and a series of experiments was therefore carried out in which the air in the solution and ampoule was replaced with nitrogen. The results obtained coincided with the reflux curve. Furthermore, the values of k obtained in the presence of nitrogen at temperatures greater than 100° C. give points which lie neatly on the extrapolation of the reflux curve. This result is not unexpected as above 70° C. (where the two curves diverge) it is not unreasonable to expect the heating to drive the dissolved air (oxygen) out of the solution and to replace it in the flask with water vapour. This will, indeed, be complete at 100° C.

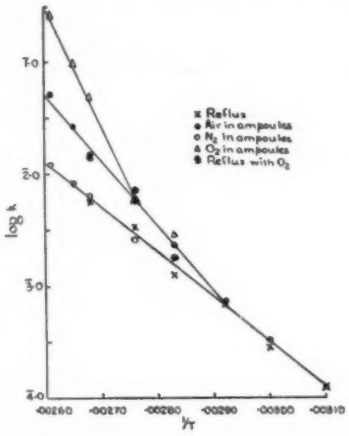

Fig. 1. log $k/1/T$ Curves for Phosphate Buffer Solutions at pH 6·5.

It then remained to determine whether the effect of oxygen was intensified by concentrations greater than that in air and a series of experiments in which the air was replaced by oxygen before the ampoules were sealed and one in which oxygen was bubbled through refluxing solutions, were carried out. The former yielded an even steeper curve which links up with the air curve at 90° C. and the latter gave points which approximate to those obtained with air sealed in the ampoules. These experiments point conclusively to the destructive effect of oxygen on aneurin, an effect which is intensified by increasing temperature.

Fig. 2 records the results obtained in a similar series of studies using McIlvaine phosphate-citric acid buffer solutions. Two points stand out. Firstly, it is clear that while O_2 is capable of accelerating the destruction of aneurin in this buffer also, and, apparently at all temperatures (the two curves are parallel), the citric acid has had the effect of reducing the sensitivity of aneurin to oxygen so that the values obtained with ampoules containing air and nitrogen, and with solutions under reflux, are all virtually the same, while those obtained with oxygen in the sealed ampoules are only slightly greater.

In Fig. 3 can be seen the results of experiments with the phosphate-phthalate buffer. The curves differ from the other series in several ways. The range of values for log k is very much smaller than in the case of the phosphate buffer and approximates to that of the phosphate-citric acid solutions. As in the case of the phosphate buffer, there is a diverging curve for ordinary refluxed solutions and almost parallel curves for air, nitrogen and oxygen sealed in

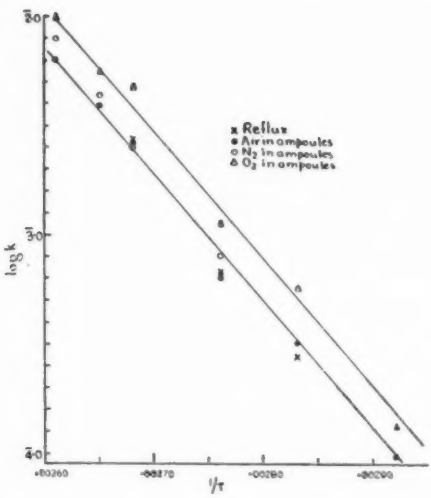

Fig. 2. log $k/1/_T$ Curves for Citric Acid Phosphate Buffer Solutions at pH 6·3.

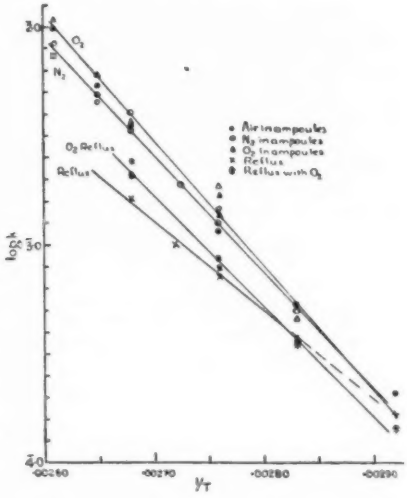

Fig. 3. log $k/1/_T$ Curves for Phosphate-Phthalate Buffer Solutions at pH 6·0.

ampoules as were obtained for the phosphate-citric acid buffer. However, these solutions all give values higher at most temperatures than those obtained for refluxed solutions with or without oxygen. Continued repetition of points on these curves, including in the work special precautions to free the nitrogen completely from traces of oxygen merely served to confirm the original results.

DISCUSSION.

In the absence of oxygen, the velocity coefficient for aneurin destruction in phosphate buffer solution is related to the temperature through the Arrhenius equation at all temperatures from 110° C. to 50° C. There appears to be no deviation from this relationship in phosphate buffer solutions at temperatures below 77° C., i.e. in the range in which Rice and Beuk (1945) did find deviations when studying the destruction of aneurin in pork. This, by inference, favours the tentative explanation of these authors that their observed deviations were due to enzyme activity or some related phenomenon. Because of the very slow rate of destruction of aneurin at the lower temperatures, it was not considered worth while studying this further with the other buffers.

Booth (1943) mentioned precautions taken to free buffer solutions from oxygen in the hope of explaining abnormally rapid destruction of aneurin. However, he could find no evidence of an oxygen effect. Apart from this there seems to be no reference in the literature to such experiments. The results

obtained with the phosphate buffer appear to represent the extreme behaviour with wide variations in the rates of destruction found and a well-defined acceleration due to oxygen. Citric acid apparently is able to moderate the oxygen effect, reducing the range of variation in the rates of destruction encountered. This sort of thing has already been noticed in earlier work (Farrer, 1947 b), where citric acid reduced the effect of the concentration of the buffer salts.

The results obtained with phosphate-phthalate solutions show reaction velocities of a similar order to those obtained with citric acid but one cannot simply ascribe a retarding effect to oxygen in the presence of phthalate ions (cf. Farrer, 1947 a, who showed that Cu could both accelerate and retard aneurin destruction according to the conditions) as the most oxygen rich solutions, those sealed in ampoules with oxygen, showed faster rates of aneurin destruction very similar at all temperatures to those found in ampoules containing air and purified (i.e. oxygen free) nitrogen. A further anomaly is the lack of agreement between the nitrogen and reflux curves. One hesitates to postulate a "pressure effect."

Further evidence of the modification (in this case it is elimination) of the oxygen effect by the constituents of the solution is found in unpublished work of the present authors on solutions of yeast extract. At 110° C., 105° C., and 100° C. oxygen, nitrogen and air sealed in ampoules with the test solutions gave the same results.

SUMMARY.

Using reaction kinetic methods employed in earlier work of a similar kind, the effect of temperature in the range 110° C. to 50° C. on the rate of thermal destruction of aneurin has been studied in three different buffer solutions; phosphate (pH 6·5), phosphate-citric acid (pH 6·3) and phosphate-phthalate (pH 6·0). There is no deviation from the Arrhenius equation under these conditions.

In using solutions sealed in ampoules to obtain temperatures above 100° C. it was found that dissolved oxygen was capable of accelerating the thermal destruction of aneurin. In phosphate solutions this was very marked and the range of velocity coefficients encountered with oxygen, air and nitrogen was large.

Citric acid greatly reduced the oxygen effect but acceleration was still observed. Only when phosphate-phthalate solutions were completely saturated with oxygen was there an indication of accelerated destruction and an anomaly was encountered in that solutions saturated with oxygen-free nitrogen showed more rapid aneurin destruction than those in contact with oxygen at atmospheric pressure.

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